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# Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry for Small Molecule Analysis in Foods

by

**Suzanne Lorraine Frison** ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**

in

**Food Science and Technology**

Department of Agricultural, Food, and Nutritional Science

Edmonton, Alberta

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Science tells us what we can know, but what we can know is little,
and if we forget how much we cannot know,
we become insensitive to many things of very great importance.

Bertrand Russell, *A History of Western Philosophy*

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry for Small Molecule Analysis in Foods** submitted by **Suzanne Lorraine Frison** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Food Science and Technology**.

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# Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a promising analytical tool in food science due to its speed, ease of sample preparation, ability to analyze complex mixtures, and simplicity of mass spectral interpretation. This thesis explores several qualitative and quantitative applications of MALDI-TOF MS in food science, focusing on key phytochemicals, polymers, and emulsifiers. Specifically, important conditions such as sample preparation, selection of matrices, use of internal standards, and determination of response factors are investigated for ginsenosides, hydrogenated starch hydrolysates, polysorbate emulsifiers, and flavonol glycosides.

A simple MALDI-TOF MS protocol to analyze ginsenosides in American ginseng root powder was evaluated by comparison with thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Ginsenosides in the biological ginseng system could not be readily analyzed by MALDI-TOF MS in that a simple purification step to separate ginsenosides from interfering substances was not achieved, mass spectra were of variable quality, and not all ginsenosides could be detected and differentiated by mass.

The composition of hydrogenated starch hydrolysates was investigated using MALDI-TOF MS. Oligomers spanning 1 to 28 units were identified, and differences in the degree of hydrolysis of a low, medium, and high maltitol syrup were observed. Spectrum-to-spectrum variability, lack of high mass standards, and mass discrimination limited the ability to quantify these polymers.

The molecular composition of polysorbate emulsifiers was studied. MALDI-TOF MS analysis before and after saponification indicated the presence of free ethylene oxide polymers, as well as free and esterified sorbitan- and sorbide-based polymers. This analysis also provided insight into the polydispersity, degree of esterification, and identity of esterified fatty acids.

Four flavonol glycosides in almond seedcoats, isorhamnetin rutinoside, isorhamnetin glucoside, kaemferol rutinoside, and kaempferol glucoside, were rapidly identified and quantified by MALDI-TOF MS. An internal standard, rutin (quercetin-3-rutinoside), was employed. Results of MALDI-TOF MS analysis were verified by HPLC. In addition, seedcoats of sixteen almond varieties were screened for flavonol glycosides. Individual peak ratios were consistent across triplicate analyses of all samples. In all almond varieties, isorhamnetin rutinoside was most abundant, and the total flavonol glycoside content ranged from 75 to 250 μg per gram of seedcoat.

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# List of Abbreviations

| | |
|---|---|
| 3-AQ | 3-aminoquinoline |
| $C_{18}$ | octadecyl silane |
| CHCA | α-cyano-4-hydroxycinnamic acid |
| CI | chemical ionization |
| DHB | 2,5-dihydroxybenzoic acid |
| DP | degree of polymerization |
| EI | electron impact |
| ESI | electrospray ionization |
| FAB | fast atom bombardment |
| FD | field-desorption |
| GC | gas chromatography |
| HLB | hydrophile-lipophile balance |
| HPAEC-PAD | high performance anion exchange chromatography with pulsed amperometric detection |
| HPLC | high performance liquid chromatography |
| HPLC-RI | high performance liquid chromatography with refractive index detection. |
| HPLC-UV | high performance liquid chromatography with ultraviolet detection |
| HSH | hydrogenated starch hydrolysates |
| LD | laser desorption |
| *m/z* | mass to charge ratio |
| MALDI | matrix-assisted laser desorption ionization |
| MS | mass spectrometry |
| PD | plasma desorption |
| PS | polysorbate |
| THAP | 2',4',6'-trihydroxyacetophenone |
| TLC | thin-layer chromatography |
| TOF | time-of-flight |

# Chapter 1

## Introduction

### A BRIEF HISTORY OF MASS SPECTROMETRY

To cover the applications of mass spectrometry would be to survey the full range of scientific endeavour in the twentieth century. It is one of the most influential research tools in recent years. Mass spectrometry has been a crucial element in developing our understanding of the natural world in such diverse areas as astronomy, geology, evolutionary biology, and archaeology. The ability of mass spectrometry to measure atomic masses to a part per billion accuracy, to monitor reactions, and to characterize natural and synthetic compounds using minute amounts of sample has transformed the field of chemistry. The high sensitivity of mass spectrometry which permits identification of trace substances in tiny samples has led to the use of mass spectrometers in toxicology, drug abuse diagnosis, metabolic studies, environmental pollution monitoring, and elsewhere (Borman, 1998). Mass spectrometers have long been an integral tool in the petroleum, chemical, and pharmaceutical industries for process monitoring and materials analysis and are now expanding into the electronics and food industries. Mass spectrometry is becoming increasingly valuable for sophisticated biomedical applications such as analysis of intact viruses and bacteria (Feneslau and Demirev, 2001), studies of immunological pathways (De Jong, 1998), and imaging (Todd et al., 2001).

Early experiments into probing the nature of matter at the turn of the nineteenth century provided the basis for the development of mass spectrometry as we know it today. Exploration of positive ray deflection patterns by J. J. Thompson provided the first evidence for the existence of stable (non-radioactive) isotopes. After World War I, improvements in resolving power and ion detection, stabilization of the experimental apparatus into routine instruments, and developments in vacuum technologies and electronics allowed expansion of mass spectrometry into new areas. During these years, mass spectrometry became instrumental in studying the history of the earth and examining the components of our solar system. In the 1940s, commercialization of mass spectrometry began with the production of magnetic deflection instruments, which were quickly adopted by the petroleum industry. During World War II, Alfred Nier and his research group used mass spectrometry to separate uranium-235 from uranium-238 and to characterize plutonium for the first time, developments which had special importance to the making of the first atomic bomb.

In the 1950s, new instrument designs began to proliferate as well as efforts to couple mass spectrometers with other types of analytical instruments such as gas and liquid chromatographs. Time-of-flight (TOF) instruments which had been proposed initially by William E. Stevens in 1946, were improved by the introduction of William C. Wiley and I.H. McLaren's time-lag focusing scheme that enhanced mass resolution by correcting for the initial spatial distribution of ions (Wiley and McLaren, 1955). Also during this time, Wolfgang Paul and his colleagues introduced the first quadrupole mass analyzer which today far exceeds the total number of other types of mass spectrometers in use (Nier, 1999).

Throughout the 1960s and into the 1970s, novel systems of generating ions extended the capabilities of mass spectrometry beyond those available with the original electron impact mass spectrometry (EIMS). Early mass spectrometry was limited mainly to analysis of atomic isotopes or light gases. Field desorption mass spectrometry (FDMS) allowed the possibility of studying compounds that were not volatile or thermally unstable. Chemical ionization mass spectrometry (CIMS) produced fewer fragment ions than electron impact, reducing the complexity of mass spectral interpretation. Diversification in mass spectrometry instrumentation in the 1980s further expanded its utility. Fast atom bombardment mass spectrometry (FAB MS) proved to be useful for obtaining spectra of large organic molecules. Plasma desorption mass spectrometry (PDMS) was shown to be feasible for studying high molecular weight proteins. Early experiments with laser desorption mass spectrometry (LDMS) began in which photon beams were used to desorb sample molecules from a solid surface.

The recent development of two ionization techniques has opened up new possibilities for mass spectrometry. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) have revolutionized the field of biotechnology. ESI mass spectrometry was first put into routine practice in the early 1980s (Whitehouse et al., 1985). Using this technique, multiply charged ions are produced with little fragmentation and biomolecules up to several hundred thousand mass units can be measured, exceeding the previous limitations of several thousand mass units. In the late 1980s, MALDI was introduced, providing a new means for soft and efficient ionization of various large, fragile, and nonvolatile biomolecules (Karas et al., 1987; Karas and Hillenkamp., 1988; Tanaka et al., 1988). This technique was ideal for coupling to a time-of-flight (TOF) mass analyzer due to the precise timing of the ionization event. The first MALDI-TOF MS instruments suffered from relatively poor mass resolution compared to the standard double-focusing magnetic sector instruments, but the advent of ion reflectors and delayed extraction in the 1990s facilitated remarkable improvements in resolving power (Brown and Lennon, 1995; Whittal and Li, 1995).

The continuous expansion of mass spectrometry capabilities throughout the last century has left virtually no scientific field untouched by its influence.

## THEORY OF MALDI-TOF MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) shares a fundamental functional characteristic with all types of mass spectrometry: the sorting of ions by mass. Implied in this description is the requirement for ion generation (ion source), separation according to mass-to-charge ratios (mass analyzer), and detection (detector). In MALDI-TOF MS, sample molecules embedded in an ultraviolet-absorbing matrix are ionized by a nanosecond laser pulse. During the process, highly energized ions and neutral molecules are ejected from the solid surface into the gas phase. After a short time delay, ions are accelerated toward the mass analyzer by an electric potential, separated by their mass-to-charge ratios during flight, and converted into electrical signals at the detector. A schematic diagram of the MALDI-TOF MS system is shown in Fig 1-1.

**Instrumentation**. The mass analyzer consists of a flight tube under high vacuum which in linear instruments is a field-free drift region and in reflector instruments involves ion deflection by electrical fields. Unlike quadrupole, ion-trap, and magnetic sector mass analyzers, TOF MS does not scan the spectrum, thus most ions entering the flight tube are detected (Guilhaus, 1995). Ions are initially imparted with similar kinetic energies but different velocities. As ions travel down the tube they separate into discrete packets according to their mass, where smaller ions travel faster and reach the detector before larger ions. The time of flight is proportional to $(m/z)^{1/2}$. Unlike ESI-MS where multiply charged ions are common, MALDI-TOF MS produces almost exclusively singly charged positive ions (Karas et al., 2000).

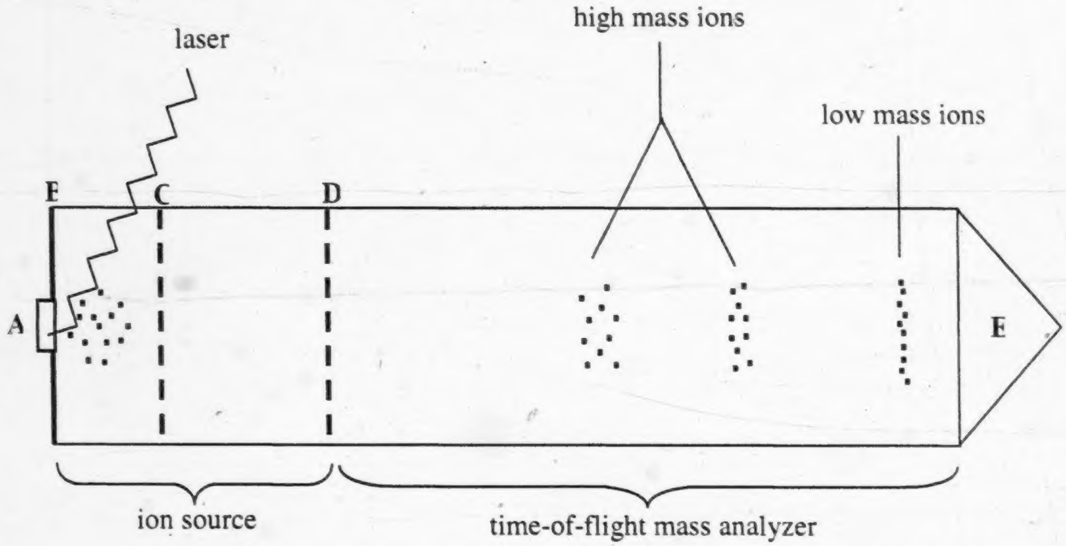


**Figure 1-1**. Schematic diagram of linear matrix-assisted laser desorption/ionization time-of-flight mass spectrometer. A: sample probe tip; B: repeller plate; C: extraction grid; D: acceleration grid; E: detector.

To achieve high mass resolution in a linear time-of-flight mass analyzer, it is necessary to correct for the initial spatial, temporal, and velocity distribution of the ions. To attain this resolution, a delayed extraction method (also known as pulsed ion extraction or time-lag focusing) is used (Whittal and Li, 1995; Brown and Lennon, 1995). A short time delay of several hundred to several thousand nanoseconds is inserted between the laser ionization and the ion extraction events, in which no fields are applied. The delay allows the energy correction necessary to focus the ions at the detector;

initially slower ions which are closest to the repeller receive the greatest energy input, and faster ions receive lower energy inputs.

**Ionization Mechanisms.** The ion formation process in MALDI-TOF MS is complex and depends on a number of factors. Formation of protonated, deprotonated, cationized and sometimes even radical species are generated relatively independently of the matrix, solvent composition, solution pH, and analyte acid-base properties (Karas et al., 2000). Empirical study has resulted in the advancement of several theories regarding possible ionization mechanisms. According to Zenobi and Knochenmuss (1998), both primary and secondary ion formation steps contribute to the outcome of a MALDI-TOF MS experiment. Primary steps refer to the initial charge separation that occurs upon laser irradiation of the sample. These include desorption of preformed ions, energy transfer between matrix and analyte molecules, proton transfer reactions, and break-up of the sample into charged clusters. Several MALDI-TOF MS ionization models presume that analyte molecules are in close contact with matrix molecules as the desorption plume is evolving (Kinsel et al., 1997; Kinsel et al., 1999; Zenobi and Knochenmuss, 1998); however, spectra generally do not exhibit ion signals corresponding to matrix adducts (Itina et al., 2002). Secondary ionization steps may completely mask primary ionization in that ions formed initially are converted into those eventually observed in the mass spectrum. These processes may include proton, cation, or electron transfer reactions, or charged particle ejection (Zenobi and Knochenmuss, 1998).

According to Karas et al. (2000), one of the most significant features of MALDI-TOF MS ionization is that only singly charged ions are observed, the vast majority of which are positively charged. Photoionization of the matrix results in loss of electrons from the plume, leaving a net positive charge. Furthermore, a dominant neutralization process occurs in the dense plume shortly after laser excitation in which highly charged species cannot survive and are reduced to a charge state of 0 or 1. As a result, ions are a minority species, existing at a concentration of about four orders of magnitude lower than neutrals (Fournier, 2002).

## CONSIDERATIONS FOR MALDI-TOF MS QUANTITATIVE ANALYSIS

MALDI-TOF MS was initially developed primarily to identify and characterize large biomolecules. Early work on MALDI-TOF MS focused on analyzing pure protein solutions (Karas et al., 1988; Karas et al., 1989). Later MALDI-TOF MS was applied to other large molecules such as peptides, carbohydrates, oligonucleotides, and synthetic polymers. Theoretically MALDI-TOF MS should be able to analyze any compound that has mass. Its capabilities now extend to many chemical classes of small molecules, polymers, hydrophobic substances, and others, and new applications continue to be developed.

MALDI-TOF MS offers a number of unique features. MALDI is known as a soft ionization technique which means that fragmentation is generally minimal. A wide range of masses can easily be detected simultaneously with high mass accuracy. Typically, MALDI-TOF MS is applied to molecules between 500 and 300 000 molecular weight, but mass determinations up to 1.5 million Daltons have been demonstrated (Schreimer and Li, 1996). Similarly, very low mass determinations are also possible. For example, Goheen et al. (1997) have generated negative ion MALDI-TOF mass spectra of small organic acids such as oxalic, fumaric, maleic, succinic and citric acids, which have *m/z* of 90, 116, 116, 118, 192, respectively. MALDI mass spectrometry also exhibits high sensitivity. Femtomolar ($10^{-15}$) determinations are common; Keller and Li (2001) have successfully applied MALDI-TOF MS to analyzing zeptomole ($10^{-18}$) quantities of large proteins using a microspot technique in which a very uniform matrix layer is applied to the target followed by capillary tube deposition of concentrated sub-nanolitre samples.

Biological samples under investigation usually contain various contaminants which play a disruptive role in detection. Compared to other types of mass spectrometry, MALDI-TOF MS is relatively insensitive to a number of impurities and is known to tolerate low levels of buffers, salts, and some denaturants (Zhang and Li, 2002; Zhang et al., 2001; Bajuk et al., 2001; Patterson and Aebersold, 1995). Unlike ESI-MS, MALDI-TOF MS has the ability to analyze complex mixtures directly, without prior extensive purification. Each peak in the spectrum (except matrix-related peaks) can often be correlated to a single component in the given mixture.

Investigation into the use of MALDI-TOF MS as a quantitative tool continues to expand its applications. To perform quantitative analysis with MALDI-TOF MS, several problems must be addressed. High concentrations of one compound may suppress the signal of less concentrated analytes (Gusev et al., 1996). Peak broadening at higher masses due to increased isotopic abundance lowers the ability to discriminate among compounds of similar masses. Fragmentation and adduct formation by cationization or protonation increase the complexity of the signal in that the total response for a given compound may be the sum of several peaks. These events also increase the probability of mass overlap. A requirement of any quantitative technique is the ability to reliably reproduce the measurements. A major limitation to accurate quantification with MALDI-TOF MS is the high spot-to-spot and sample-to-sample variability that results from poor crystal homogeneity and variable incorporation of analytes into the crystal bed. Numerous strategies have been described regarding sample preparation, selection of matrices, and use of an internal standard to improve MALDI-TOF MS reproducibility for quantitative analysis.

**Sample Preparation.** The sample preparation step is a key factor in the quality and success of MALDI-TOF mass spectrometric analysis. The original and simplest method described for MALDI-TOF MS sample preparation is referred to as the dried droplet method. It consists of mixing the analyte and matrix together in an appropriate solvent with a large molar excess of matrix (between $10^3$ and $10^5$ molar excess), depositing the solution onto the sample probe, and removing the solvent through air-drying (Bruker, 1995; Karas et al., 1988). During the process of solvent evaporation, growth of matrix crystals is induced and analyte molecules are incorporated into the crystal network. Ideally, the goal is to create a homogenous surface consisting of very small crystals where analytes are uniformly incorporated and distributed throughout. The rate of evaporation can affect the size of the crystals and the degree of analyte incorporation. Rapid evaporation creates smaller crystals and a more homogenous crystal structure, whereas slow evaporation causes larger crystals to form and may preferentially exclude analytes from the crystal lattice, in much the same way that recrystallization techniques are used to purify crystalline compounds (Trimpin et al., 2001). Large crystal formation may also be detrimental to resolution in that the non-uniform surface could cause slight variations in the distance to the detector.

Several other preparation methods have been proposed as a means to further modulate crystal formation and improve quantification. The fast evaporation method involves applying a solution of matrix saturated in a volatile solvent such as acetone to the probe first and allowing it to dry, followed by application of the sample solution (Vorm et al., 1994). Nicola et al. (1995) found that the fast evaporation method improved the point-to-point repeatability and sample-to-sample reproducibility in the quantitative analysis of angiotensin II by reducing the relative standard deviation by a factor of three compared to a standard method (multicomponent matrix). In the seed-layer method, a solution of dilute matrix is applied to the probe before depositing the sample/matrix mixture. The pre-deposited crystals act as seeds to stimulate homogenous formation of small new crystals (Onnerfjord et al., 1999). A two-layered method where the analyte/matrix mixture is deposited on top of a densely packed bed of matrix has been shown to be effective for analyzing complex mixtures of proteins and peptides (Dai et al., 1999). Electrospray deposition of matrix/protein solutions has been demonstrated to markedly improve sample homogeneity and reduce the coefficient of variation of the response factors from 49.6% to 15.1% or better when compared to the dried droplet method of sample preparation (Hensel et al., 1997).

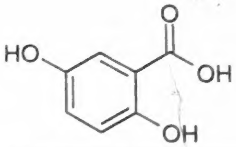
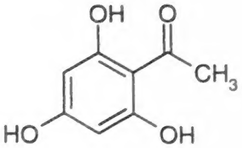
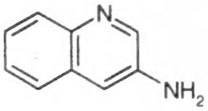
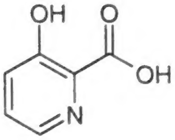
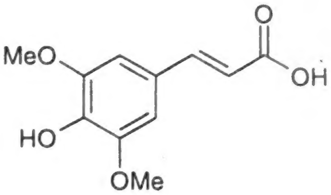
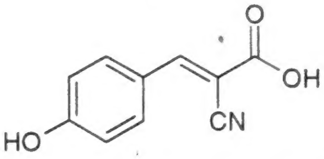
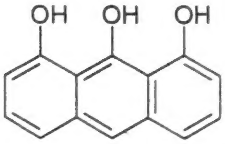
**Matrix Selection.** The choice of matrix is crucial for success in MALDI-TOF MS experimentation. The matrix protects the analytes from extensive fragmentation, assists in transforming them into the gas phase, and facilitates the ionization process. In general, MALDI-TOF MS matrices need to provide efficient ionization, controllable and reproducible fragmentation, and exhibit lack of mass interferences with the compounds of interest. Matrices employed in MALDI-TOF MS are generally small organic molecules that absorb ultraviolet light and are stable under vacuum conditions. Matrix selection and optimization is still largely a trial-and-error process, but certain generalizations can be made. When selecting matrices for specific MALDI-TOF MS applications, solubility in organic solvents and miscibility with the sample solution are important considerations (Nielen, 1999). However, solution dynamics may not necessarily be relevant in predicting the gas-phase interactions that occur between matrices and samples in the ion source. A homogenous distribution of analytes cocrystallized within the matrix is desirable to achieve consistent results. Thus, the polarity of the matrix is often roughly matched with the polarity of the analytes.

**Matrix Applications.** A variety of different matrices have been developed for a wide range of samples and approaches. Some common solid matrices are shown in Table 1-1. Matrices can be effective for analyzing certain types of samples (e.g. proteins or peptides) over other types of samples (e.g. carbohydrates or polymers), but the application boundaries for any given matrix are loosely defined. Depending on the preparation conditions, several matrices may be able to effectively analyze the same type of sample. Similarly, one matrix may be used to analyze a diverse range of samples.

2,5-Dihydroxybenzoic acid (DHB) is commonly used to analyze carbohydrates (Harvey, 1999), but isoflavones (Wang and Sporns, 2000b), oligonucleotides (Tang et al., 1997), and synthetic polyesters (Blais et al., 1995) have also been successfully detected using this matrix. 2',4',6'-Trihydroxyacetophenone monohydrate (THAP) has been used for a variety of substances including flavonol glycosides (Wang and Sporns, 2000a; Frison-Norrie and Sporns, 2002; Frison and Sporns, 2002, Chapters 5 and 6), glycoalkaloids (Driedger and Sporns, 1999), anthocyanins (Wang and Sporns, 1999), synthetic polymers (Arakawa et al., 1999), and polysorbates (Frison-Norrie and Sporns, 2001, Chapter 4). 3-Aminoquinoline has been shown to be useful for inulin (Metzger et al., 1994), fructooligosaccharides (Stahl et al., 1997), and glucosinolates (Botting et al., 2002). 3-Hydroxypicolinic acid is frequently used for oligonucleotides (Wu et al., 1993). Sinapinic acid is often used in conjunction with large proteins (Kussman et al., 1997). A relatively hydrophobic matrix, $\alpha$-cyano-4-hydroxycinnamic acid (CHCA) has been demonstrated for peptides (Kussman et al., 1997) and triglycerides (Ayorinde et al., 1999). Dithranol is a popular matrix for synthetic polymers such as poly(methyl methacrylate) and polystyrene (Kassis et al., 1997; Belu et al., 1996). Since matrix selection is largely an empirical process, new applications continue to be discovered for the common matrices, and new substances continue to be tested for their ability to function as MALDI-TOF MS matrices.

**Alternative Sample Preparations and Matrices**. Several new matrices and sample preparations have been proposed as alternatives to the typical solid crystalline

**Table 1-1**. Chemical structures of common solid MALDI-TOF MS matrices.

| **Name** | **Structure** |
|---|---|
| 2,5,-dihydroxybenzoic acid (DHB) |  |
| 2’,4’,6’-trihydroyacetophenone (THAP) |  |
| 3-aminoquinoline (AQ) |  |
| 3-hydroxypicolinic acid (HPA) |  |
| sinapinic acid (SA) |  |
| α-cyano-4-hydroxycinnamic acid (CHCA) |  |
| 1,8,9-trihydroxyanthracene (dithranol) |  |

systems. A common concern when analyzing small molecules by MALDI-TOF MS is the interference of matrix peaks in the low mass region. High molecular weight matrices such as porphyrins have been shown to be feasible for small molecule analysis in that they do not interfere in the low mass region (Jones et al., 1995). Porphyrin matrices have been successfully demonstrated for the analysis of water-soluble vitamins which have masses below 500 Daltons (Chen and Ying, 2002). Another possibility is to use a two-phase inorganic matrix system. Metal or metal oxide particles suspended in liquid paraffin or glycerol have been shown to be effective matrices for analyzing polyethylene glycol 200 and methyl stearate (Kinumi et al., 2000).

Solid crystalline matrices often suffer from poor shot-to-shot and sample-to-sample reproducibility due to the difficulty in evenly dispersing analytes throughout the solid matrix. This results in the "sweet spot" phenomenon where the analyst must scan through several positions on the probe to locate a region which generates a high-intensity signal. Armstrong et al. (2001) demonstrated that greater homogeneity and reproducibility may be achieved by the use of non-volatile UV-absorbing ionic liquids as matrices, although their ability to promote ionization may be inferior compared to solid matrices. A method of solvent-free sample preparation in which analyte, matrix, and a cationizing salt are mixed in a ball mill and applied to the probe as a powder has also been shown to improve homogeneity. Successful measurements have been made of a variety of analytes including polystyrenes, poly(methyl methacrylates), polyetherimide, C.I. Pigment Red 144, and bovine insulin (Trimpin et al., 2001). This method may prove to be a useful alternative for characterizing insoluble samples by MALDI-TOF MS.

**Selection and Use of an Internal Standard.** There are several reports that use direct signal response (Petkovic et al., 2001) or external standards (Harvey, 1993; Preston et al., 1993; Camafeita et al., 1997) to quantify using MALDI-TOF MS. However, most quantitative methods make use of an internal standard to counteract the variability in MALDI-TOF MS. Selection of an appropriate internal standard is critical to the success of the measurement. Wilkinson et al. (1997) describe the ideal internal standard as follows:

1. The internal standard must be chemically similar to the analyte. This feature implies similar extraction efficiency and incorporation into the matrix crystal structure.

2. The internal standard must be completely resolved by mass from the analyte. For small molecules, an isotopically labeled analog may suffice. However, isotopic labeling cannot be used effectively for large molecules due to peak broadening and limitations in resolving power of the linear TOF mass analyzer.
3. The internal standard must be similar in concentration to the analyte. At high analyte concentrations, competitive ionization may occur in which the signal resulting from less concentrated components is suppressed. Similarly, high internal standard concentrations may suppress the analyte signal.

Some internal standards behave similarly to the analytes in terms of adduct formation and fragmentation patterns. Fragmentation patterns which are consistent and predictable can be quantitatively determined. For example, the internal standard rutin (quercetin-3-rutinoside) was used to predict the fragmentation patterns of other flavonol glycosides in almond seedcoats for quantification (Frison-Norrie and Sporns, 2002). However, the ideal internal standard is not always available. Particularly when analyzing polymers or a group of compounds in a mixture, effects between analytes, the internal standard, and the sample environment become complicated. In this case, the assumption must be made that all analytes have the same response as that of the internal standard.

## FOOD ANALYSIS USING MALDI-TOF MS

MALDI-TOF MS is an attractive tool to apply in food science areas due to its high sensitivity, ease of sample preparation, and ability to rapidly analyze multiple compounds in complex mixtures. Prior to 1995, virtually no food analyses using MALDI-TOF MS were reported in the literature. A review published in 1998 listed only thirteen references to food analyses using MALDI-TOF MS (Sporns and Wang). Since then, MALDI-TOF MS has been tested in a wide range of food systems both qualitatively and quantitatively. The compounds analyzed in foods include proteins, peptides, carbohydrates, lipids, bacterial extracts, polyphenols, steroidal glycosides, pesticides, emulsifiers, reaction products, natural toxins and many others. A thorough review of MALDI-TOF MS and food analyses from 1995 to 1999 is provided elsewhere (Wang, 2000). An overview of food science applications which have been studied using MALDI-

TOF MS from 2000 to 2002 is shown in Table 1-2. The significant contributions of MALDI-TOF MS to food analysis since 2000 are discussed below.

**Authentication.** The ability of MALDI mass spectrometry to simultaneously analyze several compounds in a mixture highlights its potential to be used for quality control purposes. Distinct mass fingerprints in the MALDI-TOF MS spectra can be used to detect food adulteration. For example, Cozzolino et al. (2001) showed that the unique whey protein profiles of cow, ewe, and water buffalo milk in a mixture can be differentiated by MALDI-TOF MS, thus verifying their authenticity. MALDI-TOF MS has also been used to monitor the addition of sweeteners to fruit juices (Zidkova et al., 2001) and to determine the flavonol glycoside profiles of many almond varieties (Frison and Sporns, 2002).

**Food safety.** Food safety is a primary concern for consumers and manufacturers alike. The speed and sensitivity of MALDI-TOF MS have opened up possibilities for the rapid detection of bacterial biomarkers, which has been used to detect food contamination by pathogens (Holland et al., 2000). Strains of S*taphylococcus aureus,* an important human pathogen, have been differentiated by their distinct mass fingerprints (Bernardo et al., 2002). MALDI-TOF MS continues to be an important tool in identifying and characterizing certain antibacterial peptides called bacteriocins. Masses determined by MALDI-TOF MS have aided in identifying bacteriocins aureocin A53 from *S. aureus* (Netz et al., 2002), enterocins A and B from *Enterococcus faecium* isolated from cheese (Ennahar et al., 2001), and lacticin FS92 from *Lactococcus lactis* isolated from pork (Mao et al., 2001).

A further application of MALDI-TOF MS in food safety is to identify and characterize natural toxins, which are often peptides. A method has been developed to analyze ricin toxins from castor beans (Darby et al., 2001). The identification by mass of toxins connected to a fatal food poisoning incident proved the source of the toxins to be strains of *Bacillus licheniformis* (Mikkola et al., 2000). The high sensitivity of MALDI-TOF MS makes it useful for analyzing trace quantities of materials. Staphylococcal enterotoxin B was detected by a combination of surface plasmon resonance, in which toxins are captured by antibodies on the surface of a sensor chip, and MALDI-TOF MS in milk and mushroom samples to a level of 1 ng/mL (Nedelkov et al., 2000).

**Table 1-2. Summary of Food Analysis Using MALDI-TOF MS from 2000-2002**

| Group | Specifics | Reference |
|---|---|---|
| Bacteria | Identification of bacterial marker proteins in contaminated food sources | Holland et al., 2000 |
| | Discrimination of *Staphylococcus aureus* strains by mass fingerprinting | Bernardo et al., 2002 |
| Bacteriocin | Mass determination of bacteriocin from *Staphylococcus aureus* | Netz et al., 2002 |
| | Mass determination of bacteriocin from *Enterococcus faecium* | Ennahar et al., 2001 |
| | Mass determination of bacteriocin from *Lactococcus lactis* | Mao et al., 2001 |
| Reaction Products | Mass determination of glycosylated peptide products in early Maillard browning | Kislinger et al., 2002 |
| | Mass determination of glycosylated peptide products in early Maillard browning | Humeny et al., 2002 |
| | Mass determination of lactosylated β-lactoglobulin products | French et al., 2002 |
| | Mass determination of whey protein products derivatized by plant phenolic compounds | Rawel et al., 2001 |
| | Mass determination of reaction products of nisin and glutathione | Rose et al., 2002 |
| Carbohydrates | Mass screening of soybean pectic substances | Huisman et al., 2001 |
| | Mass determination of pectic substances in red beet | Strasser et al., 2002 |
| | Mass determination of soybean oligosaccharides | Huisman et al., 2000 |
| | Chain length determination and degree of branching in barley starch | Li et al., 2001 |
| | Chain length determination of debranched potato, wheat and waxy maize starch | Broberg et al., 2000 |
| | Mass determination of oligosaccharides in olive fruit | Vierhuis et al., 2001 |
| | Detection of adulteration by mass determination of starch hydrolysates in fruit juices | Zidkova et al., 2001 |
| | Mass identification and quantitative analysis of water-soluble β-glucan from barley | Jiang et al., 2000 |
| Genetic Screening | Detection of transgenic material in foods | Popping et al., 2001 |
| | Genotyping variants of β-amylase in barley | Paris et al., 2002 |
| Phytochemicals | Quantification and identification of flavonol glycosides in almond seedcoats | Frison-Norrie and Sporns, 2002 |

| | | |
|---|---|---|
| | Screening of flavonol glycosides in several almond varieties | Frison and Sporns, 2002 |
| | Identification of flavonol glycosides in tea and onions | Wang and Sporns, 2000a |
| | Identification of glucosinolates in *Brassica* spp. | Botting et al., 2002 |
| | Mass identification of isoflavones in soy products | Wang and Sporns, 2000b |
| | Quantification of anthocyanins in highbush blueberries | Wang et al., 2000 |
| | Mass determination of proanthocyanidin oligomers in grape seed extract | Krueger et al., 2000 |
| | Mass determination of proanthocyanidin oligomers in grape seed extract | Yang et al., 2000 |
| Proteins and Peptides | Mass identification and sequencing of cheese flavour peptides | Combes et al., 2002 |
| | Quantification of beta-casein fragment in cheese | Soeryapranata et al., 2002a |
| | Quantification of bitter peptide in cheese | Soeryapranata et al., 2002b |
| | Detection of milk adulteration by whey protein mass patterns | Cozzolino et al., 2001 |
| | Mass determination of gliadins in wheat | Seilmeier et al., 2001 |
| | Mass identification of barley proteins | Chmelik et al., 2002 |
| | Mass determination of denatured soy proteins | Lakemond et al., 2002 |
| | Mass determination of glycoproteins in mung bean and barley seedlings | Kotake et al., 2001 |
| | Mass identification of allergenic glycoproteins from a variety of fruits, vegetables, and nuts | Wilson et al., 2001 |
| | Mass determination of allergenic proteins in peanuts and hazelnuts | Hird, 2000 |
| | Variations in milk protein fractions | Corradini et al., 2001 |
| Lipids | Fatty acid composition of vegetable oils | Ayorinde et al., 2000 |
| | Quantitative fatty acid analysis of fats and oils | Hlongwane et al., 2001 |
| Emulsifiers | Characterization of food-grade polysorbates | Frison-Norrie and Sporns, 2001 |
| Toxins | Mass determination of staphylococcal toxins in milk and mushrooms | Nedelkov et al., 2000 |

| | | |
|---|---|---|
| | Mass identification of *Bacillus* toxins related to food poisoning | Mikkola et al., 2000 |
| | Mass identification of ricin toxin and alkaloid marker ricinine from castor beans | Darby et al., 2001 |
| Vitamins | Mass determination of water-soluble vitamins | Chen and Ling, 2002 |
| Food Packaging | Fingerprint patterns of polyethers and polyesters migrating from food packaging laminates | Lawson et al., 2000 |

A new application of MALDI-TOF MS in the food safety area is in the study of migration of food packaging components into food. Lawson et al. (2000) used a MALDI-TOF MS methodology to study the migration, into food simulants, of a series of polyurethane adhesives used in the manufacture of common food packaging materials. The unique polymeric distributions observed in the mass spectra confirmed the migration of unreacted polyether and polyester starting materials through the laminates into the food system.

**Monitoring chemical reactions.** The ability to distinguish mass differences between reactants and products allows MALDI-TOF MS to be used for tracking chemical reactions in foods and inferring the product structure. It has been used extensively to study the formation of Maillard reaction products. MALDI-TOF MS has been demonstrated to be a quick and highly selective method to study the attachment of reducing sugars to proteins early in Maillard browning (Kislinger et al., 2002; Humeny et al., 2002; French et al., 2002). Other reactions studied include the derivatization of whey proteins with plant phenolic compounds (Rawel et al., 2001) and the inactivation of nisin (a bacteriocin) by glutathione (Rose et al., 2002).

**High speed carbohydrate characterization.** Carbohydrates are structurally complex polymeric substances, varying in monomeric frameworks, branching patterns, and the natures of substituent groups. The functional properties of carbohydrates are closely related to their chemical composition. Since early in its development, MALDI-TOF MS has proven to be indispensable for rapid determination of chain length distributions in digested starch samples both qualitatively and quantitatively. For example, in the last two years MALDI-TOF MS has been used to qualitatively examine the structural features of a wide range of carbohydrate classes including pectic substances in soybeans and beets (Huisman et al., 2001; Strasser et al., 2002), and oligosaccharides in potato, wheat, and waxy maize starches (Broberg et al., 2000), barley starch (Li et al., 2001), soybeans (Huisman et al., 2000), and olives (Vierhuis et al., 2001) among others. Using a standard addition method, Jiang et al. (2000) were able to quantify β-glucans from ten varieties of barley in about 20 minutes, compared to one hour for each analysis by HPLC.

**Proteomics and high mass determination.** The proteome refers to the protein complement expressed by a genome which reflects the cellular state or the external conditions encountered by a cell (Aebersold and Goodlett, 2001). MALDI-TOF MS is an important tool in the emerging field of proteomics due to its ability to effectively analyze high mass proteins with little fragmentation. Proteins separated by gel electrophoresis can readily be analyzed by MALDI-TOF MS often with minimal sample preparation. Usually the term proteomics relates to biochemical fields, but the study of proteomics also plays an important role in food science. The systematic analysis of proteins expressed by plants is significant for variety identification, quality assessment, and breeding programs. MALDI-TOF MS characterization of wheat gliadins ranging from 36 000 to 55 000 Da has been shown to be effective for differentiating eight wheat species (Seilmeier et al., 2001) and several barley proteins including β-amylase and B3-hordein have been identified by a proteomic method combining MALDI-TOF MS, gel electrophoresis, and bioinformatics (Chmelik et al., 2002). Lakemond et al. (2002) have applied MALDI-TOF MS to study genetic variants of glycinin ranging in mass from 51 300 to 61 200 Da, which are the major seed storage protein in soybeans.

**Flavour compounds.** Many flavour compounds are peptides, which are commonly analyzed by MALDI-TOF MS. One application is in quality control during cheese manufacture. The ability to monitor flavour compounds may be useful for assessing cheese ripening or for screening cultures for debittering activity. Low molecular weight flavour peptides (500 to 3000 Da) thought to be linked to bitterness have been identified by MALDI-TOF MS in cheddar and emmentaler cheeses (Soeryapranata et al., 2002a; Soeryapranata et al., 2002b; Combes et al., 2002)

**Phytochemicals.** HPLC is a method commonly employed for isolating and identifying plant phytochemicals. In several areas, however, MALDI-TOF MS has proven to be competitive with this conventional technique. Although initially developed for the analysis of large biomolecules, MALDI-TOF MS is also practical for analyzing small molecules. Phytochemical analysis is of importance because of the potential health benefits provided by these substances. Several types of flavonoids have proven to be readily analyzed in complex food extracts by MALDI-TOF MS. Proanthocyanidins, a complex series of oligomers, have been characterized in grape seed extract by MALDI-TOF MS (Krueger et al., 2000; Yang et al., 2000). Recently, MALDI-TOF MS methodologies have been reported for identifying flavonol glycosides in tea, onions, and almond seedcoats (Wang and Sporns, 2000a; Frison-Norrie and Sporns, 2002, Chapter 5), isoflavones in soy products (Wang and Sporns, 2000b), and anthocyanins in highbush blueberries (Wang et al., 2000). Other non-flavonoid phytochemicals have been studied as well. Intact glucosinolates isolated from plant sources have been identified (Botting et al., 2002), and ginsenosides in American ginseng have been studied (Chapter 2) .

**Routine quality control.** The ability of MALDI-TOF MS to provide a "snap-shot" of the composition of a mixture allows it to be used as a rapid screening tool for quality control applications in the food industry. Compositional analysis allows comparison among similar materials and provides a basis for inferring functionality. The speed of analysis attainable by MALDI-TOF MS introduces the potential of large-scale determinations of the presence or absence of certain key components, or obtaining general impressions of mass spectral patterns. Often, MALDI-TOF MS can provide mass information which is not achievable by any other technique. For example, MALDI-TOF MS has provided unique molecular information regarding variations in the protein fractions of milk samples (Corradini et al., 2001), the flavonol glycoside content of almond seedcoats (Frison-Norrie and Sporns, 2002; Frison and Sporns, 2002 Chapters 5 and 6), the polyol content of hydrogenated starch hydrolysates (Chapter 3), and the molecular distribution of food-grade polysorbate emulsifiers (Frison-Norrie and Sporns, 2001; Chapter 4). In addition, the fatty acid compositions of various fats and oils were shown to be better resolved by MALDI-TOF MS than by GC-MS, and MALDI was more sensitive to detecting minor fatty acid components (Hlongwane et al., 2001; Ayorinde et al., 2000).

**Other applications.** MALDI-TOF MS can be used to detect allergens in foods such as a 4826 Da protein found in peanuts and hazelnuts (Hird et al., 2000), and a number of asparagine-linked glycans isolated from a collection of fruits, vegetables, cereals, legumes, and nuts (Wilson et al., 2001). Also, the ability of MALDI-TOF MS to analyze oligonucleotides has been applied to the determination of transgenic material in foods (Popping et al., 2001) and the screening of barley varieties for genetic variants of β-amylase thought to exhibit different levels of thermal stability (Paris et al., 2002).

## THESIS OBJECTIVES

The objectives of this thesis were to explore applications of MALDI-TOF MS in food science. MALDI-TOF MS has been used extensively to study large biomolecules, but despite a number of advantages, small molecules have often been overlooked by MALDI-TOF MS practitioners due to factors such as matrix-ion interference and detector saturation in the low mass range. This body of work examines several types of small molecules (<3000 Da) found in foods naturally or as additives. Food components and ingredients examined include ginsenosides, hydrogenated starch hydrolysates, polysorbate emulsifiers, and flavonol glycosides. Standards were used to model ionization and fragmentation behaviour in MALDI-TOF MS and to determine response factors for quantification. In each case, MALDI-TOF MS was evaluated as a tool for reliable determination. For each group of compounds, the research objectives were:

**MALDI-TOF MS Analysis of Ginsenosides**. To select a proper matrix for ginsenoside analysis; to study standard ginsenoside responses in MALDI-TOF MS; to develop a reliable purification scheme for ginsenosides from American ginseng root powder; to compare TLC and HPLC to qualitative MALDI-TOF results (Chapter 2).

**MALDI-TOF MS Analysis of Hydrogenated Starch Hydrolysates**. To study the responses of polyol standards in MALDI-TOF MS; to study the responses of various polyol syrups in MALDI-TOF MS; to compare MALDI-TOF MS data to manufacturer information (Chapter 3).

**MALDI-TOF MS Analysis of Polysorbate Emulsifiers**. To select a proper matrix and sample preparation conditions; to use MALDI-TOF MS as a tool to determine the molecular composition of polysorbates (Chapter 4).

**MALDI-TOF MS Quantification of Flavonol Glycosides**. To study the responses of flavonol glycosides in MALDI-TOF MS; to develop a quantitative methodology to analyze flavonol glycosides in almond seedcoats; to define quantitative fragmentation of flavonol glycosides in MALDI-TOF MS; to compare MALDI-TOF MS results to HPLC; to use MALDI-TOF MS as a screening tool to study the composition of a selection of almond varieties (Chapter 5 and Chapter 6).

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# Chapter 2

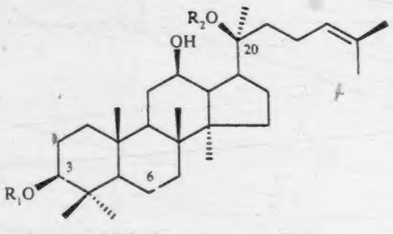
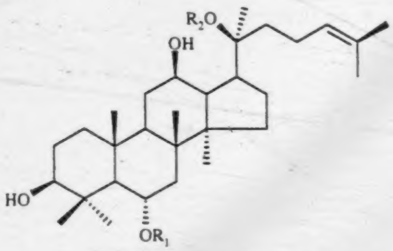
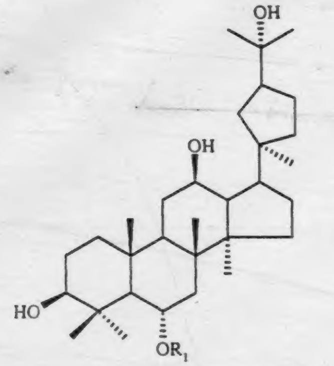
## Investigating MALDI-TOF Mass Spectrometry for Analysis of Ginsenosides in American Ginseng (*Panax quinquefolius* L.)

### INTRODUCTION

Ginseng root has been used for centuries in traditional medicine and is revered for its rejuvenating and restorative functions. The common term "ginseng" refers to at least five species of plants belonging to the genus *Panax*. The two most reknowned for their medicinal properties are Asian ginseng (*Panax ginseng* C.A.Meyer) and American ginseng (*Panax quinquefolius* L.). Asian ginseng was first documented for medicinal use over 2000 years ago, and is now one of the most popular herbs in traditional Chinese medicine (Sticher, 1998). American ginseng was discovered in North America by Europeans in the early eighteenth century, but was used by native North Americans long before the continent was colonized. American ginseng is highly prized in Asia and often demands a higher market price than Asian ginseng . Other types of ginseng, Sanchi (Tienchi), Japanese, and Vietnamese ginsengs are prepared from *Panax notoginseng* (Burkill) F.H. Chen, *Panax japonicus* C.A. Meyer, and *Panax vietnamensis* Ha et Grush., respectively.

Ginseng, like other herbal products, has a complex chemical composition which may vary considerably depending on the species, growing conditions, age of the plant, place of origin, selected portion of the plant, and the type of preparation (Soldati and Tanaka, 1984; Makhan'kov et al., 1993; Malinovskaya et al., 1993; Smith et al., 1996; Li et al. 1996, Harkey et al., 2001). All *Panax* species contain steroidal saponins known as ginsenosides, which vary mainly in the number, type, and location of their sugar moieties as shown in Table 2-1. These are the most extensively studied chemical component of ginseng and are presumed to be major active constituents (Attele et al., 1999). Ginsenosides have been shown to have a diverse range of biological activites including cognitive enhancing effects (Mook et al., 2001; Sloley et al., 1999), neuroprotective effects (Liu et al., 2001; Kim et al., 2000), immunomodulatory actions (Yu et al., 2000;

**Table 2-1.** Chemical structures of common ginsenosides.

| Structure | Ginsenoside [a] | $R_1$ [b,c] | $R_2$ [b,c] | Mol. Form. | Mol. Wt. [d] |
|---|---|---|---|---|---|
|  <br>(20*S*)-Protopanaxadiol | Rb$_1$ | -Glc$^2$-Glc | -Glc$^6$-Glc | $C_{54}H_{92}O_{23}$ | 1109.5 |
| | Rb$_2$ | -Glc$^2$-Glc | -Glc$^6$-Ara (p) | $C_{53}H_{90}O_{22}$ | 1079.4 |
| | Rc | -Glc$^2$-Glc | -Glc$^6$-Ara (f) | $C_{53}H_{90}O_{22}$ | 1079.4 |
| | Rd | -Glc$^2$-Glc | -Glc | $C_{48}H_{82}O_{18}$ | 947.3 |
| | QII | -Glc$^2$-Glc$^6$-Oc | -Glc$^6$-Glc | $C_{62}H_{104}O_{24}$ | 1233.7 |
| | mRb$_1$ | -Glc$^2$-Glc$^6$-Ma | -Glc$^6$-Glc | $C_{57}H_{94}O_{26}$ | 1195.5 |
| | mRb$_2$ | -Glc$^2$-Glc$^6$-Ma | -Glc$^6$-Ara (p) | $C_{56}H_{92}O_{25}$ | 1165.5 |
| | mRc | -Glc$^2$-Glc$^6$-Ma | -Glc$^6$-Ara (f) | $C_{56}H_{92}O_{25}$ | 1165.5 |
| | mRd | -Glc$^2$-Glc$^6$-Ma | -Glc | $C_{51}H_{84}O_{21}$ | 1033.4 |
|  <br>(20*S*)-Protopanaxatriol | Re | -Glc$^2$-Rha | -Glc | $C_{48}H_{82}O_{18}$ | 947.3 |
| | Rf | -Glc$^2$-Glc | -H | $C_{42}H_{72}O_{14}$ | 801.1 |
| | Rg$_1$ | -Glc | -Glc | $C_{42}H_{72}O_{14}$ | 801.1 |
| | Rg$_2$ | -Glc$^2$-Rha | -H | $C_{42}H_{72}O_{13}$ | 785.1 |
|  <br>(24*R*)-Pseudoginsenoside | PG-F$_{11}$ | -Glc$^2$-Rha | | $C_{42}H_{72}O_{14}$ | 801.1 |

[a] QII = quinquenoside II; PG-F$_{11}$ = pseudoginsenoside F$_{11}$; m(Rb$_1$, Rb$_2$, Rc, Rd) = malonyl(Rb$_1$, Rb$_2$, Rc, Rd)

[b] The superscripts 2 and 6 denote the positions of attachment of the terminal sugar molecule.

[c] Glc = β-D-glucose; Ara(p) = α-L-arabinopyranose; Ara(f) = α-L-arabinofuranose; Rha = α-L-rhamnose; Oc = (*E*)-2-octenoyl; Ma = malonyl

[d] Represents the average molecular weight.

Wang et al., 2000), cardiovascular effects (Gillis, 1997; Jung et al., 1998; Li et al., 2001; Yuan et al., 1999), anti-cancer properties (Matsunaga et al., 1989; Oh et al., 1999; Liu et al., 2000; Wakabayashi et al., 1998), and regulation of lipid metabolism (Yang et al., 1999). Since ginsenosides are unique to *Panax* species and are associated with pharmacologic activity, they are often used as marker compounds for quality control. Currently, more than thirty ginsenosides have been identified, the most abundant being $Rb_1$, $Rb_2$, Rc, Rd, Re, Rf, and $Rg_1$.

Concerns have arisen about the safety, purity, and potency of herbal products as they are not subject to the same labeling regulations as pharmaceuticals (Angell and Kassirer, 1998). Knowledge about their composition is required for standardization and extensive efforts toward characterization of ginseng began in the mid-1950s (Tanaka, 1994). Since then, many analytical techniques have been explored including thin-layer chromatography (Schulten and Soldati, 1981), droplet countercurrent chromatography (Otsuka et al., 1977), colourimetric techniques (Hiai et al., 1975), gas chromatography/ mass spectrometry (Cui et al., 1997), high-performance thin layer chromatography (Vanhaelen-Fastre et al., 2000), ion chromatography with pulsed amperometric detection (Park et al., 1994), infrared spectroscopy (Ren and Chen, 1999), and immunological techniques (Kanaoka et al., 1992; Fukuda et al., 1999). By far, the most common method of identifying and quantifying ginsenosides in ginseng extracts and other products is high performance liquid chromatography (HPLC) (Soldati and Sticher, 1980; Kanazawa et al., 1987; Chuang and Sheu, 1994; Court et al., 1996) or HPLC coupled with mass spectrometry (van Breeman et al., 1995; Fuzzati et al., 1999; Wang et al., 1999; Li et al., 2000; Ji et al., 2001). This combination works well in that ginsenosides of identical molecular formula can be separated chromatographically and then identified by mass, but the analysis times can be lengthy.

It has been suggested that Asian and American ginsengs can be differentiated on the basis of their relative amounts of ginsenosides. In general, American ginseng is reported to contain a greater total amount of ginsenosides than Asian ginseng (Lui and Staba, 1980; Soldati and Sticher, 1980). Ginsenosides $Rb_1$, $Rb_2$, Rc, Rd, Re, and $Rg_1$are present in both Asian and American ginseng in different proportions, whereas Rf is found almost exclusively in Asian ginseng (van Breeman et al., 1995; Li et al, 1996; Li et al., 2000). In contrast, American ginseng is reported to contain pseudoginsenoside-$F_{11}$, which is absent in Asian ginseng (van Breeman et al., 1995; Li et al, 1996; Li et al., 2000). American ginseng is reported to have a higher ratio of $Rb_1/Rg_1$ than Asian ginseng (Wang et al., 1999; Harkey et al., 2001) and a lower ratio of $Rg_1/Re$ and $Rb_2/Rc$ (Chan et al., 2000). Five new ginsenosides, called quinquenosides, have been isolated from American ginseng and may also potentially be unique markers of this species (Yoshikawa et al., 1998).

Pure standards of ginsenosides have been analyzed by electrospray ionization mass spectrometry (ESI-MS) (Miao et al., 2002) and matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Zhou et al., 1998), but mass spectrometry has not been applied to biological ginseng samples. Since its introduction in the late 1980s, MALDI-TOF MS has evolved into a technique that has recently shown promise in the area of food analysis (Sporns and Wang, 1998). Sample preparation of complex mixtures is rapid and simple, usually involving a water or alcohol extraction, followed by crude purification on a solid-phase extraction cartridge. Compounds are easily identified by molecular weight as little or no fragment ions are generated. Because samples can be analyzed in less than a minute, it was hypothesized that MALDI-TOF MS could potentially be a useful screening tool to verify the composition of various ginseng preparations. It was also postulated that MALDI-TOF MS could be used as a "fingerprinting" device to authenticate samples based on chemical profiling. Since several of the ginsenosides have identical molecular weights and cannot be differentiated by mass alone, quantification to determine relative ginsenoside ratios would be necessary for proper characterization using MALDI-TOF MS. The objectives of this study were to investigate the feasibility of MALDI-TOF MS for analyzing ginsenosides in commercial ginseng preparations using American ginseng root powder as a model.

## MATERIALS AND METHODS

**Materials and Reagents.** American ginseng root powder (*Panax quinquefolius* L.) was purchased from a local market in Edmonton, Alberta, Canada. Ginsenosides $Rb_1$,

$Rb_2$, Rc, Rd, Re, and $Rg_1$ were purchased from Extrasynthese (Genay, France). Solid-phase extraction columns (Sep-Pak $C_{18}$, $C_8$, $tC_2$, and aminopropyl, and Oasis HLB) were obtained from Waters Corporation (Milford, MA). Thin-layer chromatography plates (aluminum-backed silica gel $UV_{254}$, and glass-backed reversed phase $KC_{18}F$) were from Whatman International Ltd., Maidstone, England. 2,5-Dihydroxybenzoic acid (DHB) was obtained from Sigma Chemical Co. (St. Louis, MO) and 2',4',6'-trihydroxyacetophenone monohydrate (THAP) was purchased from Aldrich Chemical Co. (Milwaukee, WI). All water was double-deionized (Milli-Q water purification system, Millipore Corp., Bedford, MA) and all organic solvents were of HPLC grade.

**Extraction of Ginsenosides from Root Powder .** Ginsenosides were extracted from the root powder by a modification of the procedure described by Ren and Chen (1999). Fifteen milliliters of 80% methanol in water was added to 1.0 g of root powder in each of 6 centrifuge tubes; the mixture was shaken horizontally for one hour, centrifuged, and decanted through Whatman No. 1 filter paper. The extraction was repeated twice more, combining the supernatants. Methanol was removed on a rotary evaporator with bath temperature of 30°C. The volume of the yellow extract was adjusted to approximately 30 mL with water and stored at 4°C until use.

**Purification by Solid-Phase Extraction .** Aqueous ginseng extract was filtered through a Millex-GS 22μm membrane (Millipore Products Division, Bedford, MA) prior to solid-phase extraction. Several types of Sep-Pak cartridges were tested, including $tC_2$, $C_8$, $C_{18}$, and aminopropyl ($NH_2$). The procedure for Sep-Pak $tC_2$ and Sep-Pak $C_8$ extraction was as follows: cartridges were preconditioned with 2 mL methanol then 5 mL water, ca. 10 mL of extract was loaded onto the column, the column was washed with 2 x 5 mL 30% methanol in water and eluted with 2 x 5 mL 70% methanol in water. The Sep-Pak $tC_2$ was eluted further with 5 mL methanol. Extraction using the Sep-Pak $NH_2$ cartridge was a modification of the method reported by Li et al. (1996). The aqueous extract (ca. 10 mL) was loaded onto a Sep-Pak $C_{18}$ cartridge (preconditioned with 2 mL methanol and 5 mL water) and washed with 5 mL 30% methanol in water. Ginsenosides were eluted with 5 mL methanol into a tandem Sep-Pak $NH_2$ cartridge, preconditioned with 5 mL water and 5 mL methanol. The cartridges were eluted with 1 mL methanol. Six types of conditions were tested with the Sep-Pak $C_{18}$ cartridge, as shown in Table 2-

2. In general, aqueous ginseng extract was loaded onto the preconditioned column, washed with water or dilute methanol, ethanol, or acetonitrile, and eluted with aqueous methanol, ethanol, or acetonitrile.

**Table 2-2**. Summary of solid phase extraction protocols tested on Sep-Pak $C_{18}$ cartridges for ginseng sample preparation.

| **Conditions** | A | B | C |
|---|---|---|---|
| **No. of Experiments** | 6 | 2 | 1 |
| **Sample Volume** | 5, 8 mL | 8, 10 mL | 2 mL |
| **Wash** | 10 mL water | 5, 10 mL water | 4 mL 60% $CH_3CN$ |
| **Fraction 1** | 0, 5, 2x5, 4x5 mL 30% MeOH | 2x5 mL 30% EtOH | 2 mL 70% $CH_3CN$ |
| **Fraction 2** | 5, 4x5 mL 80% MeOH | 0, 5 mL 50% EtOH | (2x2) mL 90% $CH_3CN$ |
| **Fraction 3** | 5, 2x5 mL MeOH | 5, 2x5 mL 80% EtOH | |
| **Fraction 4** | | 5 mL EtOH | |

| **Conditions** | D | E | F |
|---|---|---|---|
| **No. of Experiments** | 3 | 2 | 11 |
| **Sample Volume** | 5 mL | 2 mL | 2, 3 mL |
| **Wash** | 0, 2 mL 20% MeOH | 5, 3x3 mL 30% MeOH | 0, 5 mL water |
| **Fraction 1** | 0, 4 mL 60% MeOH | 0, 2 mL 70% MeOH | 4 mL 60% MeOH |
| **Fraction 2** | 2 mL 70% MeOH | 2, 2x2 mL 90% MeOH | 2 mL 70% MeOH |
| **Fraction 3** | 0, 2, 2x2 mL MEW[a] (7:2:1) | | 2, 2x2, 5, 6, 4+2, 3x2, 5x2 mL 90% MeOH |
| **Fraction 4** | 0, 2, 3x2 mL MEW[a] (6:3:1) | | |

[a] methanol:ethanol:water

A mini-column packed with 2.5 mg of Oasis HLB material was fashioned from a 200 μL pipet tip. A few grains of sand in the tip held the packing in place. The column was preconditioned with 50 μL methanol then 50 μL water. Forty microlitres of a 1:1 aqueous solution of ginsenosides $Rb_1$ and $Rg_1$ (0.5 mg/mL) was applied. The column was washed and eluted in successive 30 μL fractions of 20%, 40%, 60%, 80%, and 100% methanol. Twice, the aqueous ginseng extract (200 μL) was loaded onto a fresh column, washed with 30 μL of 40% methanol, and eluted sequentially with 30 μL of 60%, 80%, and 100% methanol.

**Thin-Layer Chromatography (TLC).** Both silica gel and reversed-phase TLC plate systems were tested for separating and detecting ginsenosides in a mixture. Standards and test solutions were separated on silica gel plates using n-butanol:ethyl acetate:water (15:1:4, upper phase). When applied to the reversed-phase plates, 80% methanol was used as the eluent. Plates were visualized by charring with 5% sulfuric acid in ethanol, or by $UV_{254}$ absorbance.

**MALDI-TOF Mass Spectrometry.** MALDI-TOF MS analyses were performed using a Bruker Proflex III in linear mode (Bruker Analytical Systems, Inc., Billerica, MA). The sample solution and a solution of DHB (10 mg/mL in 10% ethanol) were mixed in a 1:1 ratio, and 1 µL was applied to a MALDI-TOF MS probe and allowed to air-dry. Alternatively, 1 µL of a saturated solution of THAP in acetone was spotted onto the probe and air-dried, followed by 1 µL of sample solution applied on top of the matrix. Ginsenosides cocrystallized with the matrix were ionized by a nitrogen laser pulse at 337 nm and accelerated under 20 kV with time-delayed extraction before entering the time-of-flight mass spectrometer. Laser strength was attenuated to obtain optimal resolution and signal-to-noise ratios. Each spectrum was obtained in the positive ion mode.

**High Performance Liquid Chromatography (HPLC).** HPLC analyses were performed on a Waters 2690 separations module equipped with an autoinjector and a Waters 486 UV detector. Separations were carried out using a Supelcosil LC-18 reversed phase column (15 cm x 4.6 mm, 5 µm, Supelco, Bellefonte, PA) preceded by a 50 x 4.6mm guard column containing Supelco LC-18 reversed-phase packing (20-40 µm, Supelco, Bellefonte, PA). Solvent A was either phosphate buffer (2.80 g $K_2HPO_4$ in 2000 mL HPLC grade water, pH adjusted to 5.8 with $H_3PO_4$) or water; solvent B was acetonitrile. The flow rate was 1 mL/min with a linear gradient profile consisting of solvent A with the following proportions (v/v) of solvent B: 0 min, 15% B; 25 min, 25% B; 37 min, 32% B; 45 min, 40% B; 52 min, 90% B; 55 min, 15% B. Injection volume was 10 µL, detection was at 205 nm, and total run time was 60 minutes. A Shimadzu CLASS-VP chromatography data system (Shimadzu Scientific Instruments Inc., Columbia, MD) was used to monitor the eluted peaks.

## RESULTS AND DISCUSSION

**Monitoring the Ginseng Extract by Thin-Layer Chromatography.** A thin-layer chromatography system was used to monitor ginsenosides in the various fractions during the exploration of solid-phase extraction methods. In addition to ginsenosides, which appeared as pinkish-purple spots on the silica plates after charring, other yellowish-brown spots were often visible. Up to twelve compounds were resolved by silica TLC in the ginseng extract eluted in 60% methanol from the Sep-Pak $C_{18}$, five of which were tentatively identified as ginsenosides on the basis of colour and retention factor comparison with standards. The ginsenosides were invisible by UV detection, whereas several of the impurities were visible under $UV_{254}$. On the silica plates, the yellowish impurities tended to stay near the baseline, whereas on the reversed-phase plates, they traveled with the solvent front; in both cases, the ginsenosides remained near the centre of the plate. For these reasons, the yellowish impurities appeared to be more polar than the ginsenosides. Silica TLC was the preferred system for tracking ginsenosides as the developing time was shorter and the resolution was superior to the reversed-phase plates.

TLC revealed that neither ginsenosides nor yellowish impurities were eluted from the $C_{18}$ Sep-Pak using 30% methanol, whereas both ginsenosides and yellowish impurities were eluted using 2 mL or more of 60%, 70%, 80%, or 90% methanol. Ginsenosides were not reliably detected by MALDI-TOF MS in fractions which contained the yellowish impurities. Washing with several column volumes of aqueous methanol often removed enough of the interfering substances to obtain MALDI-TOF MS spectra of ginsenosides, though with variable results, as ginsenosides were also removed during the process. For example, TLC analysis of a 60% methanol wash showed the presence of ginsenosides plus yellowish impurities, but no ginsenosides were visible in the mass spectrum. In the mass spectrum of subsequent fractions eluted in 70% or 90% methanol, ginsenosides were frequently visible, though the mass profiles, peak ratios, and strength of response were variable. None of the many solid-phase extraction conditions tested were able to reliably produce clean samples for MALDI-TOF MS analysis. The Oasis HLB columns behaved similarly to the Sep-Pak $C_{18}$ in that samples purified using these columns could not be reproducibly analyzed by MALDI-TOF MS.

**Ginsenoside Analysis by High Performance Liquid Chromatography.** An HPLC protocol, currently the most common method of ginsenoside analysis, was employed to be able to monitor ginsenosides more definitively than TLC. As shown in Figure 2-1a, ten ginsenosides were identified by comparing the retention times to those of standards, as well as by comparison with previously published data (Ren and Chen, 1999; Court et al., 1996; Li et al., 1996). When phosphate buffer was changed to water, peaks

3-6 migrated to an earlier retention time (Figure 2-1b). This migration in the absence of buffer is evidence that these peaks likely represent four malonyl ginsenosides (mass spectral data also provide evidence for malonyl ginsenosides). Due to their acidic nature, the malonyl derivatives are more sensitive to changes in pH than their neutral counterparts and their chromatographic behaviour changed accordingly. It is interesting to note that the chromatographic profile of the malonyl ginsenosides mirrors the profile of their neutral counterparts. As with the TLC methodology, chromatographic analysis by HPLC clearly confirmed the presence of ginsenosides in certain fractions from solid-phase extraction in which the ginsenoside response was non-existent by MALDI-TOF MS analysis.

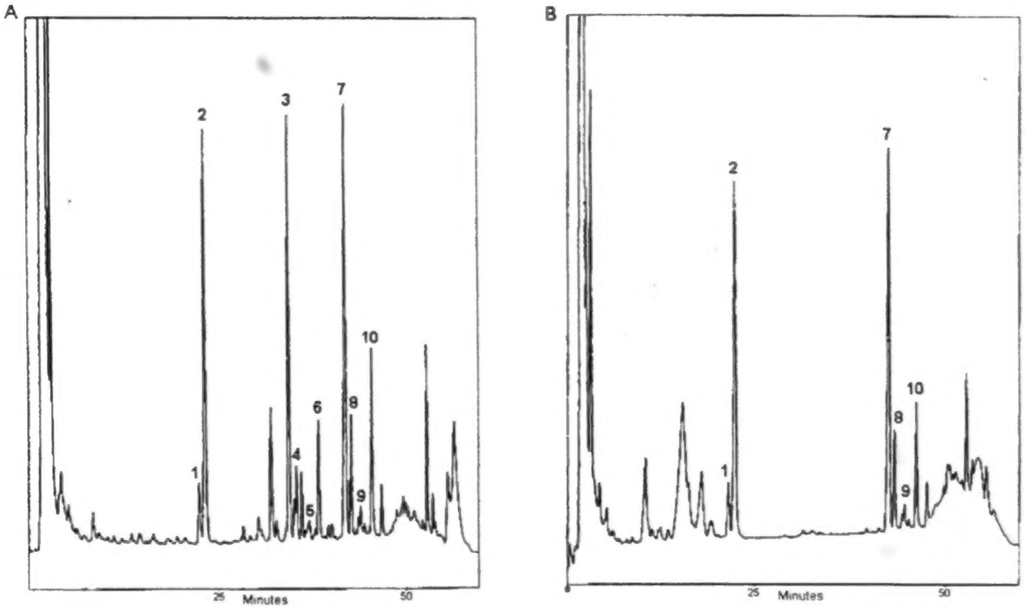


**Figure 2-1.** Typical HPLC chromatograms of ginseng extract monitored at 205 nm where Solvent A is a) phosphate buffer or b) water. 1- $Rg_1$, 2-Re, 3-$mRb_1$, 4-mRc, 5-$mRb_2$, 6-mRd, 7-$Rb_1$, 8-Rc, 9-$Rb_2$, 10-Rd.

**MALDI-TOF Mass Spectrometry of the Ginseng Extract.** Two common matrices were examined for MALDI-TOF MS desorption and ionization of ginsenosides. Ginsenoside standards analyzed in the positive ion mode with matrices 2',4',6'-trihydroxyacetophenone monohydrate (THAP) or 2,5-dihydroxybenzoic acid (DHB) produced strong responses. However, sensitivity and reproducibility were somewhat better with DHB, thus this matrix was used for all subsequent analyses.

As shown in Figure 2-2, ginsenoside ions were in the form of single alkali metal adducts $[M+Na]^+$ or $[M+K]^+$, where the sodium adducts are dominant. Fragmentation with loss of carbohydrate residues is common in MALDI-TOF MS. Since ginsenosides contain multiple sugar residues, fragment ion peaks corresponding to sequential carbohydrate losses might be expected. In fact, only a single fragment ion peak at *m/z* 790 was observed for standards of $Rb_1$, $Rb_2$, Rc, Rd, and Re, which differ in molecular weight and the number and type of associated sugars. This peak is consistent with the loss of the carbohydrate residue at position 20. Because no other fragment ions were observed, it seems that fragmentation occurs only at this location. When analyzing the ginseng extract, another type of fragmentation was observed where the most abundant

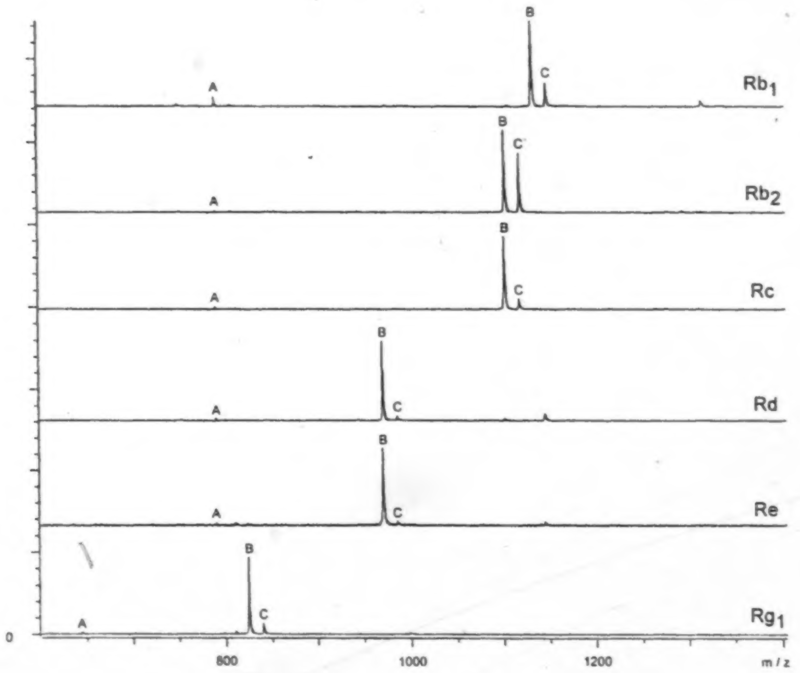


**Figure 2-2.** MALDI-TOF MS spectra in positive ion mode of ginsenoside standards $Rb_1$, $Rb_2$, Rc, Rd, Re, and $Rg_1$ dissolved at 1 mg/mL in 50% aqueous methanol. A: $[M - (\text{sugar at C20}) + H]^+$; B: $[M+Na]^+$; C: $[M+K]^+$.

malonyl ginsenoside, mRb1, produced fragment ions corresponding to loss of $CO_2$. This type of fragmentation did not occur for the other malonyl ginsenosides, presumably because they were less abundant.

Mass spectra of the ginseng extract were variable. Often ginsenoside responses were weak and of low resolution and appeared across several fractions from the solid-phase extraction columns. As shown in Figure 2-3, preparation conditions which did produce spectra with high response and resolution were not consistently reproducible. Even under identical preparation conditions, the masses and types of adducts observed varied from one time to the next. Table 2-3 identifies all of the ginsenosides detected in the ginseng sample at various times throughout the study, though not all were observed simultaneously in any one spectrum. Several ion forms were observed for many of the ginsenosides. Of the three analytical methods employed, MALDI-TOF MS was the only one to identify quinquenoside II in the sample.

It is possible that the sample extract was too complex to be able to easily isolate ginsenosides adequately to allow for MALDI-TOF MS identification. Ginsenoside response may have been suppressed by the presence of other compounds in the extract. One possible source of interference is flavonol glycosides which are yellow in colour, extracted and eluted under the same conditions as ginsenosides, and known to exist in ginseng (Liu and Xiao, 1992). However, they were not detected in this study by MALDI-TOF MS, even though they have been easily detectable in other complex food matrices such as onions, tea, and almonds (Wang and Sporns, 2000; Frison-Norrie and Sporns, 2002, Chapter 5). To do a more extensive preliminary purification using a technique such as HPLC would negate the ease of sample preparation and speed of analysis, which are important advantages of MALDI-TOF MS.

In addition to problems with consistency, MALDI-TOF MS analysis of ginsenosides is inherently limited in that several ginsenosides are indistinguishable by mass (Table 2-1), including the marker compounds Rf and pseudoginsenoside-$F_{11}$ which are unique to Asian and American ginseng respectively. MALDI-TOF MS would therefore not be useful to differentiate these two species on that basis. Another reason complete compositional analysis of ginsenosides in ginseng is not possible by MALDI-TOF MS is that minor ginsenosides known to exist in American ginseng

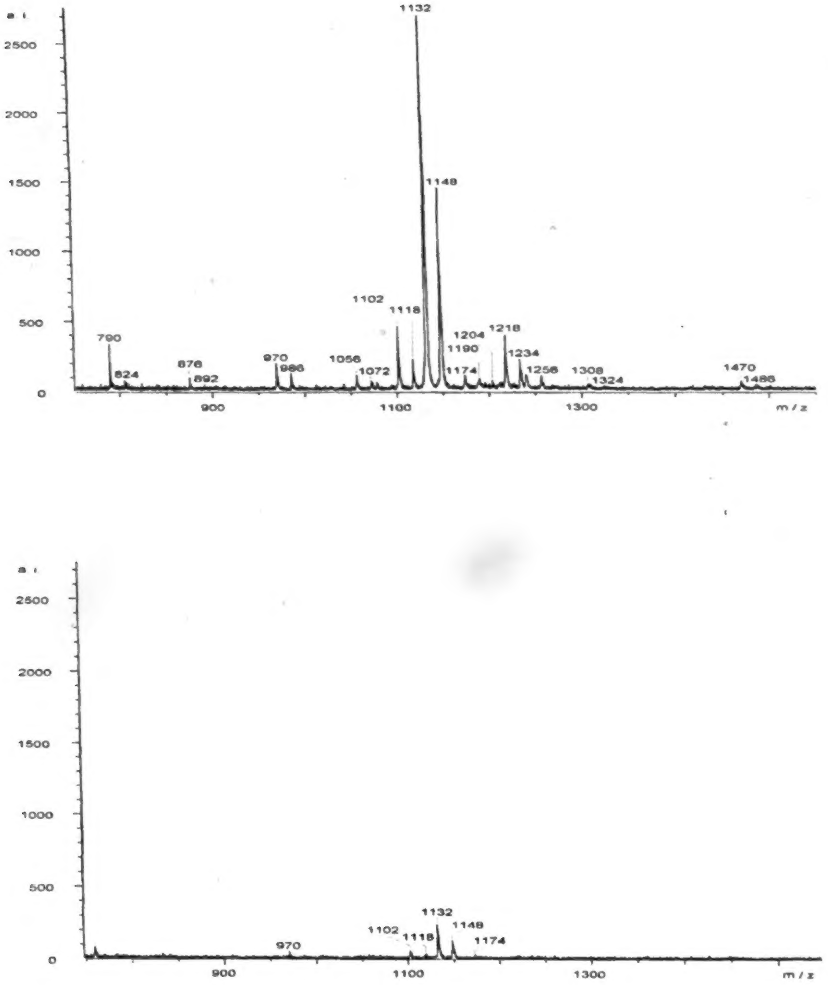


**Figure 2-3.** MALDI-TOF MS spectra of the 90% methanol eluates of two replicates of ginseng extract purified in the same manner on a Sep-Pak $C_{18}$ cartridge. Aqueous ginseng extract (2 mL) was applied to the cartridge, and washed with 4 mL of 60% methanol and 2 mL of 70% methanol prior to being eluted with 2 mL of 90% methanol. See Table 2-3 for peak identification. Peaks at *m/z* 1308, 1324, 1470, and 1486 are unidentified.

**Table 2-3**. Molecular weight assignments of peaks observed in the MALDI-TOF MS spectra of ginsenosides.

| **Ginsenoside**[a] | **Theoretical MW** | **Observed MW** | **Adducts** |
|---|---|---|---|
| **$Rb_1$** | 1109.5 | 1132 | $[M+Na]^+$ |
| | | 1148 | $[M+K]^+$ |
| | | 790 | $[M-162-180+Na]^+$ |
| **$Rb_2$, Rc** | 1079.4 | 1102 | $[M+Na]^+$ |
| | | 1118 | $[M+K]^+$ |
| | | 790 | $[M-162-150+Na]^+$ |
| **Rd, Re** | 947.3 | 970 | $[M+Na]^+$ |
| | | 986 | $[M+K]^+$ |
| | | 790 | $[M-180+Na]^+$ |
| **$Rg_1$, Rf, PG-$F_{11}$** | 801.1 | 824 | $[M+Na]^+$ |
| | | 840 | $[M+K]^+$ |
| **ma-$Rb_1$** | 1195.5 | 1218 | $[M+Na]^+$ |
| | | 1234 | $[M+K]^+$ |
| | | 1174 | $[M-44+Na]^+$ |
| | | 1190 | $[M-44+K]^+$ |
| | | 876 | $[M-162-180+Na]^+$ |
| | | 892 | $[M-162-180+K]^+$ |
| **ma-$Rb_2$, ma-Rc** | 1165.5 | 1188 | $[M+Na]^+$ |
| | | 1204 | $[M+K]^+$ |
| | | 876 | $[M-162-150+Na]^+$ |
| | | 892 | $[M-162-150+K]^+$ |
| **ma-Rd** | 1033.4 | 1056 | $[M+Na]^+$ |
| | | 1072 | $[M+K]^+$ |
| | | 876 | $[M-180+Na]^+$ |
| | | 892 | $[M-180+K]^+$ |
| **QII** | 1233.7 | 1256 | $[M+Na]^+$ |
| | | 1272 | $[M+K]^+$ |

[a] PG = pseudoginsenoside; ma = malonyl; Q = quinquenoside

(ginsenosides-$Rg_2$, -$F_2$, pseudoginsenoside-$RC_1$, quinquenosides-I, -III, -IV, -V, gypenoside XVII, notoginsenosides-A, -C, -K ) are not detected. Furthermore, it is not useful to use this MALDI-TOF MS protocol to screen samples, due to poor reproducibility and therefore inability to quantify. Thus, differentiation of common ginseng species on the basis of ginsenoside ratios would not be feasible using this MALDI-TOF MS method.

This study illustrates a situation where the limitations of the MALDI-TOF MS technique were too restrictive to recommend its use as an analytical tool in a complex food matrix. Although analysis of ginsenoside standards looked promising and provided insights into fragmentation patterns, MALDI-TOF MS was less successful when applied to the problem of ginsenoside analysis in a ginseng sample. The protocol described here lacked a reliable and simple procedure for sample clean-up, could not achieve reproducible spectra with high resolution and strong responses, and was not capable of detecting and differentiating all of the ginsenosides, all of which are necessary components of food analysis by MALDI-TOF MS.

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# Chapter 3

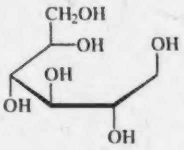
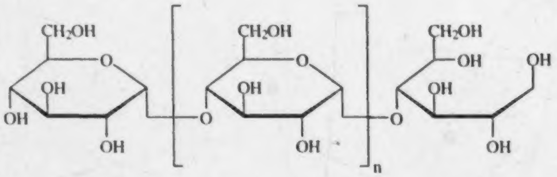
## Characterization of Hydrogenated Starch Hydrolysates Using MALDI-TOF Mass Spectrometry

### INTRODUCTION

Hydrogenated starch hydrolysates (HSH) are a family of bulk sweeteners used in a variety of foods, including confectionary products, baked goods, and frozen desserts (Calorie Control Council, 2002). They are a low-calorie substitute for sugar, exhibit a reduced glycemic response, and do not promote dental caries. For these reasons, they are often used in diet food formulations, diabetic products, and chewing gums. Although they are not as sweet as sucrose, they are often used synergistically with other sweeteners to achieve the desired taste of the product (Calorie Control Council, 2002). HSH are syrups produced by hydrogenation of the saccharide products of the partial hydrolysis of starch. The end product is a mixture of hydrogenated saccharides known as polyols or sugar alcohols, exhibiting varying degrees of polymerization (Table 3-1.). By varying the conditions and extent of hydrolysis, different formulations can be achieved to fulfill certain functional roles with respect to levels of sweetness, viscosity, or water-binding capacity.

Because the nature of HSH products and their applications depends on the molecular composition, accurate characterization is important. There are no reports to date of compositional analysis of HSH, but individual polyols have been analyzed by several chromatographic methods. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been commonly used as a sensitive method to determine small sugar alcohols without prior derivatization both in pure form and in foods (Ball, 1989; Martens and Frankenberger, 1990 and 1991; Paskach et al., 1991; Dionex Corp., 1995; Corradini et al., 1997). Quantification of several disaccharide alcohols has also been achieved using this method (Cataldi et al., 1999). Mono- and disaccharide alcohols have been analyzed in confectioneries by high performance liquid chromatography with ultraviolet detection (HPLC-UV) (Nojiri et al., 2000), but derivitazation is required to facilitate the ultraviolet absorbance. HSH manufacturers currently employ size-exclusion high performance liquid chromatography with refractive index detection (HPLC-RI) to determine the relative distribution of polyols in HSH (SPI Polyols Inc., New Castle, DE). No derivatization is required for this method, but it is much less sensitive than HPAEC-PAD (Martens and Frankenberger, 1990). All of these chromatographic methods require at least a half-hour per sample run and are unable to resolve large polymers.

**Table 3-1**. Chemical structures of common polyols and hydrogenated starch hydrolysates.

| | n | **Polyol** |
|---|---|---|
|  | | sorbitol (DP-1) |
|  | 0<br>1<br>2<br>n | maltitol (DP-2)<br>maltotriitol (DP-3)<br>maltotetraitol (DP-4)<br>hydrogenated starch hydrolysate |

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a promising technique to characterize HSH. Since its development in the late 1980s, MALDI-TOF MS has evolved into a versatile tool for characterization of natural and synthetic polymers. Attractive features of MALDI-TOF MS include its high sensitivity, speed of analysis, and ability to analyze complex mixtures. MALDI-TOF MS is ideal for analyzing polymers because, unlike chromatography, it can easily resolve the units of a homologous series by mass, and can analyze even large polymers of up to 500 kDa (Kazmeier, 1998). Several families of oligosaccharides have successfully been analyzed by MALDI-TOF MS, including isomaltooligosaccharides (Vinogradov and Bock, 1998), fructooligosaccharides (Losso and Nakai, 1997; Wang et al., 1999b), and maltooligosaccharides (Kazmaier et al., 1998; Wang et al., 1999a). Since HSH are the hydrogenated relatives of the maltooligosaccharide series, analysis by MALDI-TOF MS should theoretically be possible. In this study, the feasibility of MALDI-TOF MS for characterizing HSH is investigated for the first time.

## MATERIALS AND METHODS

**Materials and Reagents.** HSH were provided by SPI Polyols Inc. (New Castle, DE) and Lonza Inc. (Fair Lawn, NJ). Product examples of low, medium, and high maltitol syrups (8%, 20%, and 59% maltitol) were Hystar 6075, Hystar 3375, and SPI Polyols 123-5, respectively. Maltitol, maltotriitol, and maltotetraitol standards were obtained from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan). Sorbitol, maltopentaose, maltohexaose, and maltoheptaose were acquired from Sigma Chemical Co. (St. Louis, MO). 2,5-Dihydroxybenzoic was purchased from Sigma Chemical Co. and 2',4',6'-trihydroxyacetophenone monohydrate (THAP) and 3-aminoquinoline (3-AQ), were obtained from Aldrich Chemical Co. (Milwaukee, WI). Sugarless candies (Maltee™) were purchased from a local grocery store. All water was double-deionized (Milli-Q water purification system, Millipore Corp., Bedford, MA). All other reagents were of analytical grade.

**Preparation of Maltopentaitol (DP-5), Maltohexaitol (DP-6), and Maltoheptaitol (DP-7).** Maltopentaose, maltohexaose, and maltoheptaose (20 mg) were each dissolved in 10 mL water. Sodium borohydride (ca. 5 mg) was added to each solution which was stirred at room temperature for 2 hours. When the reaction was complete, the solution was acidified with acetic acid to destroy the excess sodium borohydride. Formation of maltopentaitol, maltohexaitol, and maltohexaitol was verified by MALDI-TOF MS.

**Preparation of HSH and Sugarless Candy Solutions.** Commercial HSH were dissolved in aqueous 0.01 M NaCl at ca. 5 mg/mL. Candies were manually crushed and dissolved in 0.01M NaCl at a concentration of ca. 20 mg/mL.

**MALDI-TOF Mass Spectrometry.** MALDI-TOF MS analysis was performed using a Proflex™ III instrument with a linear flight tube (Bruker Analytical Systems Inc., Billerica, MA). 2,5-Dihydroxybenzoic acid matrix solution (10 mg/mL in 50% ethanol) was mixed 1:1 with sample solutions, and a 2 µL aliquot was spotted on the probe and allowed to air-dry. Polyols co-crystallized with the matrix were ionized by a nitrogen laser pulse (337 nm) and accelerated under 20 kV with time-delayed extraction before entering the time-of-flight mass spectrometer. Laser attenuation was adjusted to optimize the signal-to-noise ratio, and samples were analyzed in the positive ion mode. All spectra were acquired as the sum of 180 laser pulses.

## RESULTS AND DISCUSSION

The performance of three matrices commonly used for carbohydrate analysis by MALDI-TOF MS was tested with respect to polyols. An equimolar standard mixture of maltitol (DP-2), maltotriitol (DP-3), and maltotetraitol (DP-4) was prepared and analyzed in conjunction with the matrices 2',4',6'-trihydroxyacetophenone monohydrate (THAP), 3-aminoquinoline (AQ), or 2,5-dihydroxybenzoic acid (DHB) (Figure 3-1). Of the three matrices, DHB demonstrated the best spot-to-spot reproducibility and produced spectra with the highest signal-to-noise ratios, and so was chosen for all subsequent analyses. No fragmentation was observed. The choice of matrix influenced the relative responses of the three polyol standards. With AQ and DHB, the polyol responses increased as the molecular weight increased, whereas the opposite was true for THAP, an occurrence also noted by Wang et al. (1999a) when analyzing the related maltooligosaccharides. When an equimolar mixture of standards ranging from DP-2 to DP-7 was tested with each of these matrices, only DHB produced a suitable spectrum with good response for all six standards; spectra obtained with AQ and THAP did not indicate the presence of any polyol peaks. These matrices may not facilitate polyol ionization as easily as DHB.

As illustrated in Figure 3-2., a solution of polyol standards DP-2 through DP-7 at equivalent weight concentrations analyzed with DHB showed a decrease in response as mass/degree of polymerization increased, whereas at equivalent molar concentrations, the reverse was true. This finding is in opposition with reports on the analogous

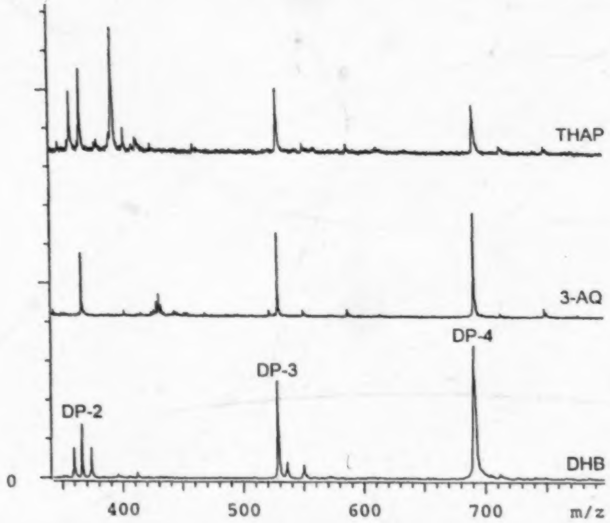


**Figure 3-1.** MALDI-TOF MS positive ion spectra of polyol standards with three matrices. A mixture of maltitol (DP-2), maltotriitol (DP-3), and maltotetraitol (DP-4) at equimolar concentrations (1.0 mM) was prepared in 0.01 M aqueous NaCl.

maltooligosaccharides by Kazmaier et al. (1998), where the responses of equal weight concentrations of maltose (DP-2) and maltotriose (DP-3) were much lower than those of maltohexaose (DP-6) and maltoheptaose (DP-7). However, this result does agree with a report by Wang et al. (1999a), where equimolar concentrations of maltooligosaccharides DP-3 through DP-7 exhibited higher responses as mass increased. The observed responses cannot be confidently correlated with the amounts of each compound when a mass or molar basis for the response relationship is not obvious.

The response relationship itself also was not clear, which poses a problem for quantification, as one cannot predict the response factors of the higher molecular weight polyols based on the smaller ones. In theory it is possible to circumvent this problem by determining the response factors of each individual polyol, or observing response trends in a series of representative examples of various masses spanning the range of detection (assuming there would be a trend), but polyol standards larger than DP-4 are not readily available. Because Wang et al. (1999a) were able to quantify maltooligosaccharides in

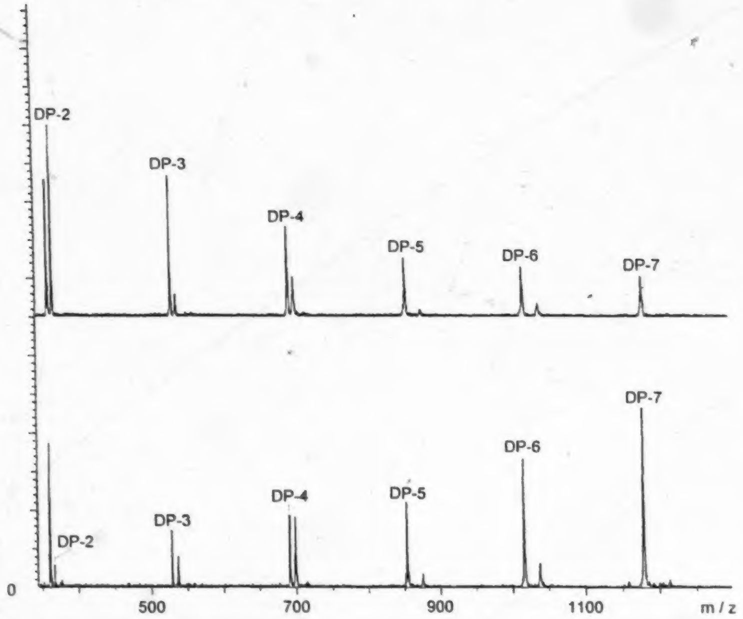


**Figure 3-2**. MALDI-TOF MS positive ion spectra of a mixture of polyol standards (DP-2 through DP-7) dissolved in 0.01 M aqueous NaCl with DHB matrix. Top: equal mass concentrations (0.3 mg/mL); Bottom: eqimolar concentrations (0.4 mM). All labeled peaks are $[M+Na]^+$. Secondary peaks 8 *m/z* higher than DP-3 and DP-4 are unidentified. Secondary peaks 22 *m/z* higher than DP-5 and DP-6 are identified as $[M + 2Na - H]^+$.

hydrolyzed starch based on their relative responses, more research is needed to determine whether polyol mixtures behave similarly to their maltooligosaccharide counterparts.

Compared to proteins of similar size, small oligosaccharides often exhibit lower ionization efficiency in MALDI-TOF MS (Harvey, 1999). This is because oligosaccharides lack basic sites for protonation; as a result, they tend to ionize by the addition of metal cations, usually $Na^+$, with low efficiency. Derivatization of oligosaccharides by incorporating neutral basic sites or cationic sites has met with varying degrees of success (Harvey, 1999). Introduction of a quaternary ammonium centre ("quaternization") into both reducing and non-reducing saccharides, including sorbitol, has been shown to increase sensitivity by as much as 1000-fold (Gouw et al., 2002). The same group of researchers reported an intense MALDI-TOF MS peak from 10

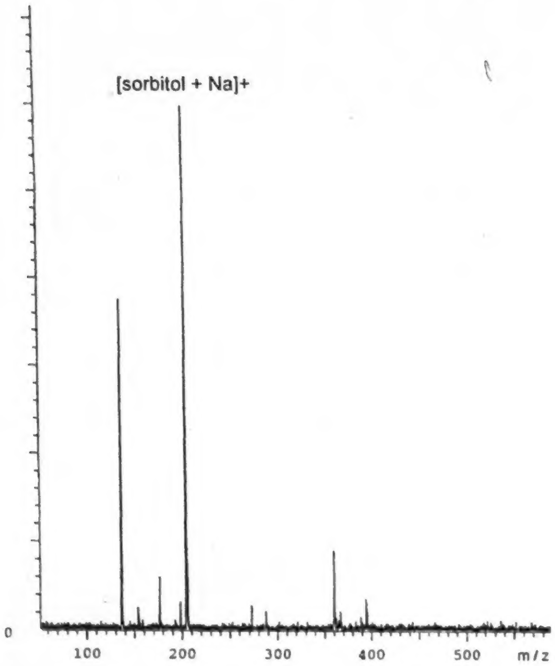


**Figure 3-3**. MALDI-TOF MS positive ion spectrum of 0.5 μg of sorbitol in water with DHB matrix.

ng of quaternized sorbitol. For comparison, in the same study 10 ng of glucose produced a response barely discernible from the background noise. Figure 3-3 shows a MALDI-TOF MS spectrum of 0.5 μg of sorbitol prepared in water. In contrast to the results of Gouw et al., these results indicate that derivatization is not necessary to obtain a strong response from small sugar alcohols in MALDI-TOF MS, provided that the concentration is high enough. However, to achieve high sensitivity at lower concentrations, derivatization may be necessary.

Three HSH syrups with varying degrees of hydrolysis were studied using MALDI-TOF MS. The mass spectra of a representative low (8%), medium (20%), and high (59%) maltitol syrup are shown in Figure 3-4. Ten replicate spectra were obtained for each sample and responses of individual species were determined relative to the total response. Degrees of polymerization from 1 to 28 units were detected, which is the highest mass known to be resolved in HSH characterization. In contrast, manufacturer characterization of the syrups by high performance liquid chromatography with refractive index detection (HPLC-RI) could only resolve up to DP-10. Sorbitol or maltitol consistently showed the strongest response. A second crest in response occurred at either DP-6 or DP-7, depending on the sample, which was corroborated by data from the manufacturers. Syrups of very different compositions can easily be differentiated by MALDI-TOF MS: a trend appears in Figure 3-4 where differences in the degree of hydrolysis of the low, medium, and high maltitol syrups are clearly discernible. The percent standard devations of the total response of ten replicate spectra were 11, 15, and

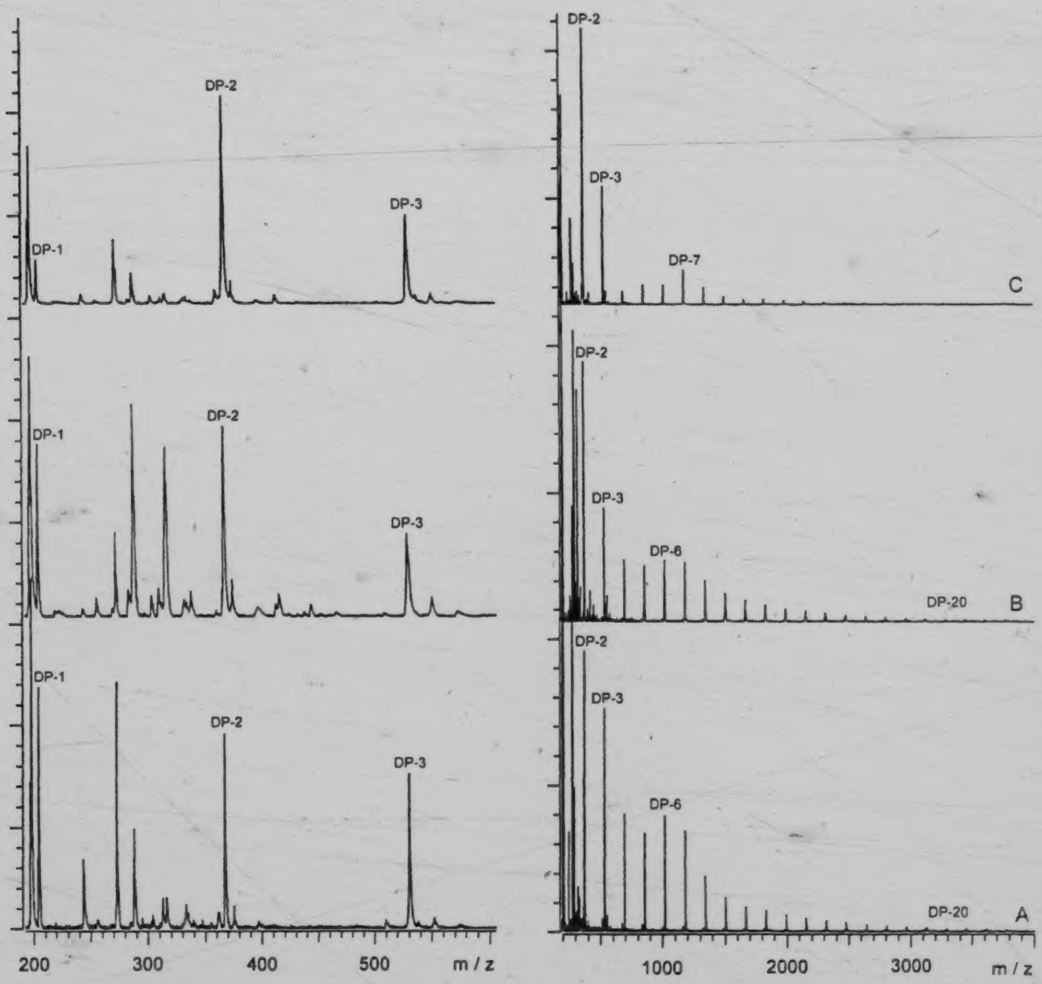


**Figure 3-4**. MALDI-TOF MS positive ion spectra of a) low, b) medium, and c) high maltitol syrups. Left: expansion of mass region *m/z* 200-500. All sample peaks are $[M+Na]^+$ ions. Extraneous peaks at *m/z* <500 are from the DHB matrix.

20 for the low, medium, and high maltitol syrups, respectively. In other words, there may be as much as a 20% variation between trials in the peak profile of a given hydrogenated starch hydrolysate, which must be taken into consideration when attempting to discriminate between syrups of more similar compositions.

Wang et al. (1999a) reported responses for maltooligosaccharides in MALDI-TOF MS to be directly related to the relative composition of the debranched starch mixture. In contrast, a variety of HSH exhibited mass profiles which did not correlate well with HPLC-RI data from the manufacturers (Table 3-2). A number of factors may account for this discrepancy. MALDI-TOF MS can definitively identify oligomers of any size based on their mass, whereas HPLC-RI cannot resolve high mass polymers and identifies oligomers indirectly. Both MALDI-TOF MS and HPLC data are subject to a degree of variability; it is not known how the two methods compare on this basis. MALDI-TOF MS may be subject to mass discrimination effects, where molecules in a certain mass range may be preferentially ionized. Considering all of these factors, a direct

**Table 3-2**. Comparison of percent response of hydrogenated starch hydrolysates determined by MALDI-TOF MS (*MS*) or as indicated on product specification sheets (*HPLC*).[1]

| | Low Maltitol Syrup | | Medium Maltitol Syrup | | High Maltitol Syrup | |
|---|---|---|---|---|---|---|
| **DP** | **MS** | **HPLC** | **MS** | **HPLC** | **MS** | **HPLC** |
| 1 | 24.1 +/- 3.6 | 14 | 23.6 +/- 3.1 | 15 | 10.3 +/- 1.2 | 7 |
| 2 | 19.1 +/- 1.5 | 8 | 26.9 +/- 4.1 | 20 | 37.6 +/- 7.0 | 59 |
| 3 | 13.8 +/- 0.7 | 10 | 13.0 +/- 0.9 | 11 | 22.9 +/- 2.5 | 14 |
| 4+ | 43.4 +/- 5.3 | 68 | 36.9 +/- 6.6 | 54 | 4.0 +/- 1.3 | 2 |
| 5 | | | | | 5.0 +/- 1.1 | 2 |
| 6 | | | | | 4.2 +/- 1.0 | 2 |
| 7 | | | | | 6.4 +/- 1.8 | 3 |
| 8 | | | | | 3.2 +/- 1.3 | 2 |
| 9 | | | | | 1.5 +/- 0.6 | 0 |
| 10 | | | | | 1.0 +/- 0.5 | 0 |
| 11+ | | | | | 4.8 +/- 1.9 | 8 |

[1] Low and medium maltitol syrups were from Lonza Inc.; these data were resolved on product specification sheets up to DP-3. High maltitol syrup was from SPI Polyols Inc.; these data were resolved on product specification sheets up to DP-10. MS results are the average of ten spectra.

percent response comparison between MALDI-TOF MS and HPLC data is not meaningful beyond crude estimates without understanding the relationships between response and concentration. High mass standards would be needed to elucidate these relationships to compare the two methods of analysis more accurately.

Excess sodium (0.01 M NaCl) added to all of the polyol solutions enhanced the formation of sodium adduct ions and suppressed the formation of potassium adduct ions. In the case of maltooligosaccharides dissolved in water, Wang and coworkers (1999a) showed that both sodium and potassium adducts were present and that potassium adducts became dominant over sodium adducts as mass/degree of polymerization increased. This switch in adduct dominance occurred at DP-23. For this reason, it was hypothesized that potassium ions may enhance the signal of high mass polyols. To test this theory, mass spectra were obtained of the low maltitol syrup dissolved in water and 0.01M aqueous KCl. Dissolved in water, both sodium and potassium adduct peaks appeared for all observed masses, but unlike maltooligosaccharides, the sodium adduct peak always dominated. Furthermore, when analyzed in the presence of excess potassium ions, only masses up to DP-9 responded (data not shown). From these observations, it seems that potassium does not necessarily enhance the response of higher mass polyols, or possibly that the effect does not become significant until reaching a certain critical mass range.

The determination of a polymer distribution should be possible if the homologues of the polymer are uniformly dissolved and homogenously distributed in the crystallized matrix. All of the polyol homologues are very soluble in water, however there may be differential crystallization with the matrix leading to preferential detection of smaller homologues.

An investigation into the composition of a sugarless candy was carried out.[1] Polyols up to DP-13 were detected with good resolution. However, because of the difficulties in correlating the MALDI-TOF MS response with the actual percent composition, this information offers little additional insight into the relative amounts of each type of polyol present.

In this study, MALDI-TOF MS provided valuable qualitative information about the nature of hydrogenated starch hydrolysates, with its ability to rapidly identify a broad array of distinct polyols in a complex mixture, including resolution of high molecular weight polyols up to DP-28 for the first time. Although the mass profiles do not correlate well to the composition reported by the manufacturers, no standards are available to validate either analytical method (i.e. direct comparison of percent response may not be relevant to actual amounts). If high mass polyols could be obtained in pure form to study the response and mass discrimination effects between high and low mass homologues, it would be possible to develop a method for quantification using MALDI-TOF MS. It may be useful to validate the MALDI-TOF MS method by separating polyols on HPAEC-PAD rather than HPLC, because it exhibits greater sensitivity and resolving ability. Appropriate response factors could then be determined and the concentration profiles compared to those determined by mass spectrometry. Further study is also needed to examine why HSH behave differently in MALDI-TOF MS than their analogous maltooligosaccharide counterparts. Overall, MALDI-TOF MS has potential to be a screening tool for characterizing HSH and serving quality control functions.

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[1] Analysis of sugarless candy was carried out by Kristina Marvin.

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# Chapter 4

## Investigating the Molecular Heterogeneity of Polysorbate Emulsifiers Using MALDI-TOF Mass Spectrometry[1]

### INTRODUCTION

Polysorbates are non-ionic emulsifiers comprised of the partial fatty acid esters of sorbitol-derived cyclic ethers (sorbitans and sorbides) condensed with approximately 20 moles of ethylene oxide per mole. Although sorbitan esters themselves exhibit emulsifying properties, polymerization with ethylene oxide improves their water solubility, thereby expanding their applications. Currently, three types of polysorbates are approved for food use in Canada: polysorbate 60, polysorbate 65, and polysorbate 80 (Health Canada, 2000). In the United States, polysorbate 20 is also permitted (FDA, 2000). These types of additives are found in a wide variety of food products, including ice cream, salad dressings, cake mixes, coffee whiteners, alcoholic cocktails, and breath freshener products. Levels of addition are generally restricted to less than 0.7%, a limit which varies depending on the product.

The capacity of polysorbate emulsifiers to perform particular functions in foods is dependent on their chemical nature, as dictated by the structure of the sorbitol derivative core, the alkyl chain length of the fatty acids, the degree of esterification, and the number of polymerized oxyethylene residues. For example, both the size of the hydrophilic headgroup and the size of the lipophilic tailgroup have been shown to influence the degree of lipid oxidation that occurs in oil-in-water emulsions (Silvestre et al., 2000; Chaiyasit et al., 2000). Understanding the chemical nature is the key to predicting properties, and by extension, to selecting emulsifiers to suit many different applications.

The molecular heterogeneity of polysorbates presents an analytical challenge. Since the introduction of the hydrophile-lipophile balance (HLB) system several decades ago, few attempts have been made to further elucidate the nature of polysorbate molecular complexity, although many analytical methods have been developed. Techniques such as colorimetry (Daniels et al., 1982; Kato et al., 1989), infrared spectroscopy (Kato et al., 1989), and gravimetric determination by precipitation (Smullin et al., 1971; AOAC Method 974.11) focus on qualitative detection of polysorbates in food products. Quantification and rudimentary characterization have been attempted using techniques such as thin-layer chromatography (Daniels et al., 1982; Kato et al., 1989; Murphy, 1969; Cumme et al., 1997), gas chromatography (Kato et al., 1989; Lundquist et al., 1971; Lindner et al., 1974), high performance liquid chromatography (Cumme et al., 1997; Tani et al., 1997), $OH^-$ negative ion chemical ionization mass spectrometry (Brumley et al., 1985) and recently, an optical chemical sensor method (Yang et al., 2000). Unfortunately, none of these methods allows satisfactory separation or resolution of the individual polymer species nor identification of the components. Several of these methods require saponification prior to analysis, which destroys and simplifies the original composition of the heterogeneous emulsifiers, and all of these methods can be laborious and time-consuming. In a recent report where matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to analyze polyoxyethylene-type emulsifiers (Cumme et al., 1997), the spectra were of poor quality and peak identity was not addressed.

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[1] A version of this chapter has been published. Frison-Norrie, S.; Sporns, P. Investigating the molecular heterogeneity of polysorbate emulsifiers by MALDI-TOF MS. *J. Agric. Food Chem.* **2001**, *49*, 3335-3340.

The technique known as MALDI-TOF MS was first demonstrated in 1987 (Karas et al., 1987), and though still in its infancy with regard to food analysis, shows great potential towards a variety of analytical problems (Sporns and Wang, 1998). Its advantages over other methodologies include ease of sample preparation, speed of analysis, high sensitivity, and minimal fragmentation allowing direct access to molecular weight. A limitation, however, is the inability to differentiate between compounds of the same molecular weight. Very recently, MALDI-TOF MS has been successfully applied to analyze other types of carbohydrate-based polymers found in foods such as fructooligosaccharides (Wang and Sporns, 1999) and maltooligosaccharides (Wang et al., 1999). This study describes a simple method to determine, for the first time, the molecular complexity of polysorbate emulsifiers using MALDI-TOF MS. The objectives of this research were to select a proper matrix for polysorbates and to identify the polysorbate molecular components before and after saponification by means of their mass profile.

## MATERIALS AND METHODS

**Materials and Reagents.** Samples of Tween™ 60K (polyoxyethylene (20) sorbitan monostearate; polysorbate 60) and Tween™ 80K (polyoxyethylene (20) sorbitan monooleate; polysorbate 80) were donated by Quest International (Lachine, Quebec, Canada). 2',4',6'-Trihydroxyacetophenone monohydrate and 3-aminoquinoline, were obtained from Aldrich Chemical Co. (Milwaukee, WI). 4-Hydroxy-α-cyanocinnamic acid was purchased from Sigma Chemical Co. (St. Louis, MO). All water was double-deionized (Milli-Q water purification system, Millipore Corp., Bedford, MA), and all reagents and were of analytical grade.

**Saponification.** Saponification was carried out as described in the literature (AOAC Method 974.11). Each polysorbate sample (200 mg) was refluxed with 12 mL of 1 M ethanolic KOH. After 45 min. of reflux, 2.5 mL of water was added to the reaction flask. Reflux was continued for an additional 15 min.. The mixture was then transferred to a separatory funnel containing 12 mL of water. The solution was acidified with concentrated HCl, mixed, allowed to cool, and extracted twice with 12 mL of hexane. The aqueous layer was desalted by swirling with a mixed-bed ion exchange resin (TMD-8, Sigma Chemical Co.) until some of the blue indicator colour remained in the resin. The resin was washed with 40 mL of water. The wash was combined with the aqueous layer and the solution evaporated to dryness using a rotary evaporator at 40°C. The polyoxyethylene sorbitan (carbowax) residue was reconstituted in 40 mL of aqueous 0.01M potassium chloride to give ca. 5 mg/mL concentration for MALDI-TOF MS analysis.

**Methyl Esterification and Gas Chromatography (GC) of Fatty Acid Fraction.**[1] The method of methyl esterification and gas chromatography of the fatty acid fraction was a variation of a standard procedure (AOAC Method 996.06). Polysorbates

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[1] Gas chromatography of fatty acid methyl esters was performed by Gary Sedgwick.

(10 to 20 mg) were dissolved in 5 mL of boron trifluoride reagent (14% $BF_3$ in methanol:methanol:hexane (7:9:4 v/v)) and heated for 30 minutes. Fatty acid methyl esters were extracted by shaking with 4 mL of water and 4 mL of hexane. The hexane layer was diluted 1:10 for GC analysis.

**MALDI-TOF MS.** MALDI-TOF MS analysis was performed using a Proflex III instrument with a linear flight tube (Bruker Analytical Systems Inc., Billerica, MA). External mass calibration was preformed using maltotetraose and angiotensin II standards. To prepare the probe, 1 μL of 2',4',6'-trihydroxyacetophenone saturated in acetone was applied and allowed to air-dry. Then 1.5 μL of the polysorbate solution (5 mg/mL in aqueous 0.01 M potassium chloride) was spotted onto the matrix crystals and further air-dried. The polyoxyethylene sorbitan esters were ionized by a nitrogen laser pulse (337 nm) and accelerated under 20 kV with time-delayed extraction before entering the time-of-flight mass spectrometer. Laser attenuation was adjusted to optimize the signal-to-noise ratio, and samples were analyzed in the positive ion mode. Spectra were acquired as the sum of 200 laser shots.

## RESULTS AND DISCUSSION

The degree and type of esterification and the number of oxyethylene residues described in commercial names of polysorbate products reflect an estimation of the dominant species. For example, polysorbate 60 is commercially known as polyoxyethylene (20) sorbitan monostearate even though the molecular species to which this name refers is only one member of a family of related compounds. Complete polysorbate analysis presents a challenge, as there are several factors contributing to molecular heterogeneity. In the preliminary steps of polysorbate manufacture, mixtures of fatty acids are heated (generally at 225 to 250°C) with sorbitol in the presence of an acid catalyst (Chislett and Walford, 1976) (Figure 4-1). These conditions drive the dehydration reaction of sorbitol, whereby one water molecule may be lost to produce cyclic sorbitol ethers known as sorbitans, or two water molecules may be lost to produce cyclic sorbitol diethers known as sorbides (Markley, 1961) (Figure 4-2). Under these conditions, virtually no acyclic sorbitol would remain in the final product (Baur, 1973).

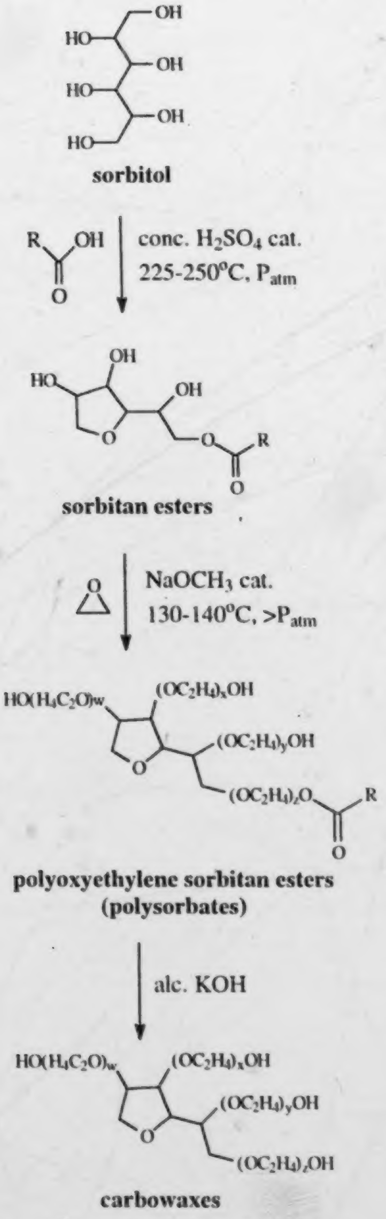
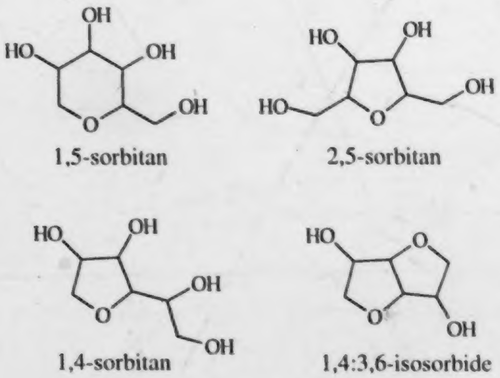

sorbitol
conc. $H_2SO_4$ cat.
225-250°C, $P_{atm}$
sorbitan esters
$NaOCH_3$ cat.
130-140°C, $>P_{atm}$
polyoxyethylene sorbitan esters
(polysorbates)
alc. KOH
carbowaxes

**Figure 4-1.** Synthesis and saponification of polysorbates.

Following cyclization, esterification occurs (Brown, 1943), probably on the primary alcohol due to steric effects (Baur, 1973). Subsequent condensation of sorbitan esters with ethylene oxide at high pressure in the presence of an alkaline catalyst favours rapid interesterification, essentially scrambling the fatty acid moieties among many possible sites. The result is a complex mixture of esters and partial esters of sorbitans and sorbides, with varying degrees of esterification, and varying alkyl chain lengths of the fatty acids, exhibiting the polydispersity characteristic of any polymeric species. Furthermore, due to the asymmetric nature of sorbitol, many stereoisomers would also be produced. MALDI-TOF MS can elucidate the presence of sorbitan-, sorbide-, and disorbitan-based species, the polydispersity of ethylene oxide chains, the degree of esterification, and the types of esterified fatty acids.


1,5-sorbitan

2,5-sorbitan

1,4-sorbitan

1,4:3,6-isosorbide

**Figure 4-2.** Structures of cyclic sorbitol-derived ethers.

Three common matrices were examined for desorption and ionization of polysorbates. Of these matrices, 2',4',6'-trihydroxyacetophenone monohydrate, produced the highest quality spectra. It easily facilitated desorption and ionization, and demonstrated good spot-to-spot repeatability and high resolution. 4-Hydroxy-α-cyanocinnamic acid saturated in acetonitrile: 0.01% trifluoroacetic acid (1:2) also produced high quality spectra, but sample preparation was more laborious. 3-Aminoquinoline was not suitable as response was low and resolution poor.

Dissolving the samples in aqueous 0.01 M potassium chloride assisted in sample ionization and simplified the spectra by enhancing the potassium adduct peaks to the exclusion of other adduct peaks (particularly $[M+Na]^+$). Species were resolved exclusively as potassium adducts with *m/z* values ranging approximately from 400 to 3000. Peaks in any given series are separated by a mass of 44.1, corresponding to the difference of one ethylene oxide residue. The polydispersity of the ethylene oxide polymer is responsible for the observed Gaussian distributions (Figures 4-3 and 4-4).

The composition of fatty acids in each sample was confirmed by GC analysis of the corresponding fatty acid methyl esters. Stearate and palmitate were identified in PS 60, in approximately a 1:1 ratio. Present in PS 80 were oleate, stearate, and palmitate, in roughly an 8:1:1 proportion.

Although the degree of esterification can be controlled somewhat by the molar ratios of reactants during production, ultimately a probability distribution occurs. The spectra shown in Figure 4-3 reflect the complexity introduced by the varying degrees of esterification and different types of fatty acids. Complete compositional analysis of PS 60 and PS 80 was unachievable due to limitations inherent in a mass spectrometric analysis, which is unable to differentiate among compounds with the same or similar molecular weights. Peak identities by mass included unesterified polyoxyethylenes, sorbides, and sorbitans, monesterified sorbides and sorbitans, diesterified sorbitans, and trace amounts of disorbitan-based species. Many of the peaks had several possible identities since several plausible molecules matched the observed molecular weights (Table 4-1). Thus, the observed pattern is the sum of all of the possible components, with the relative contributions from each individual species unknown.

To simplify the problem, fatty acids were removed from the polysorbates by saponification. Complete hydrolysis was confirmed by no change in the MALDI-TOF MS spectra following two subsequent rounds of re-saponification. Examination of spectra acquired after saponification (Figure 4-4) revealed four distinct molecular series which form the core in the absence of fatty acids: polyoxyethylenes (simple linear chains not bound to sorbitol-derived ethers), isosorbide carbowaxes, sorbitan carbowaxes, and trace amounts of species speculated to be disorbitan carbowaxes. Often, species present in trace amounts exhibit poor resolution, resulting in molecular weight "drift". Thus, identification of trace species is tentative when relying solely on molecular weight. These compounds appear in both carbowax spectra in an approximate ratio of 1 : 1 : 2 : 0.2. The fact that both the PS 60 and PS 80 spectral distributions are essentially the same shape after saponification confirms that the esterified fatty acids are indeed a defining attribute of various polysorbate formulations.

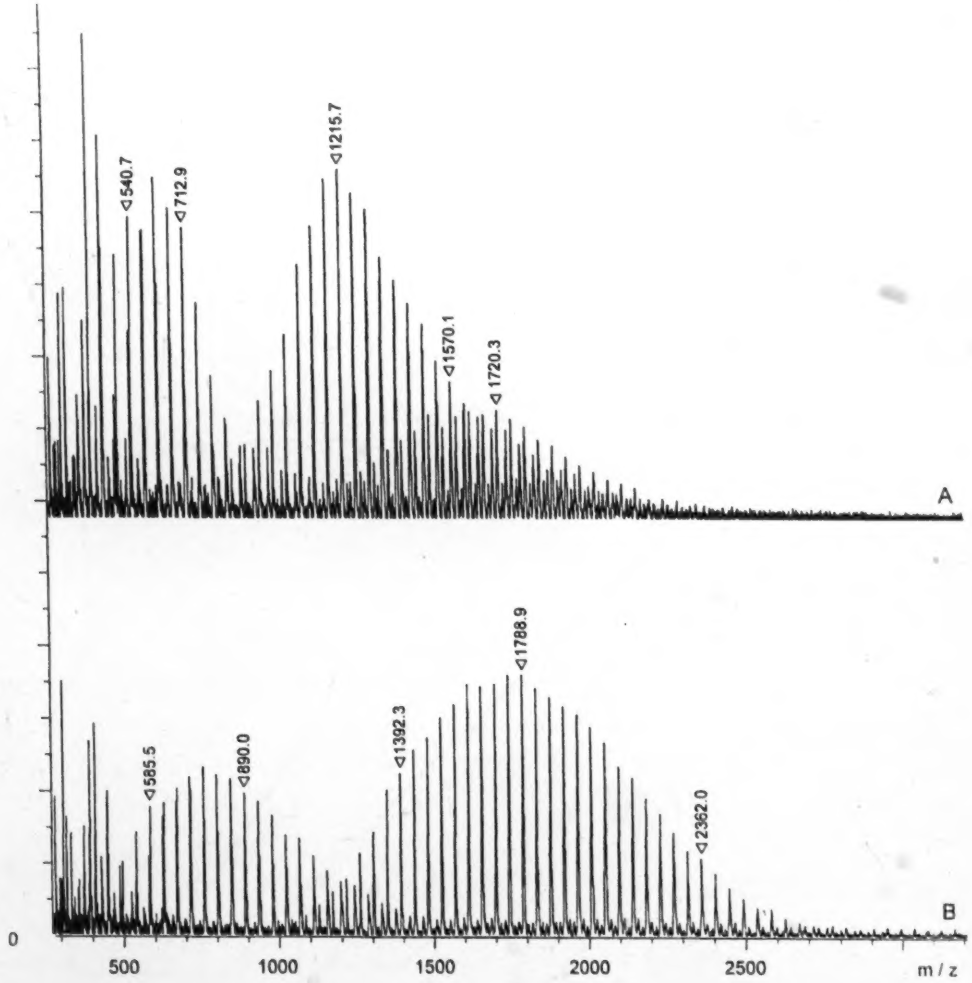


**Figure 4-3.** MALDI-TOF MS positive ion spectra of PS 60 (a) and PS 80 (b) at 5 mg/mL in aqueous 0.01 M potassium chloride. All major peaks correspond to potassium adducts. For identification of labeled peaks, see Table 4-1. Extraneous peaks below *m/z* 500 result from fragmentation of the matrix.

**Table 4-1.** Identification of polysorbate peaks labeled in Figure 4-3.

| Sample | Observed MW | Possible Identities | Theoretical MW |
|---|---|---|---|
| **PS 60**<br>(Fig. 4-3a) | 540.7 | polyoxyethylene (11) | 541.6 |
| | 712.9 | polyoxyethylene (12) isosorbide | 713.7 |
| | 1215.7 | polyoxyethylene (17) isosorbide monopalmitate<br>polyoxyethylene (23) sorbitan | 1216.3<br>1216.4 |
| | 1570.1 | polyoxyethylene (25) sorbitan monostearate | 1570.8 |
| | 1720.3 | polyoxyethylene (29) sorbitan monopalmitate | 1719.0 |
| **PS 80**<br>(Fig. 4-3b) | 585.5 | polyoxyethylene (12) | 585.7 |
| | 890.0 | polyoxyethylene (16) isosorbide | 890.0 |
| | 1392.3 | polyoxyethylene (22) isosorbide monopalmitate<br>polyoxyethylene (27) sorbitan<br>polyoxyethylene (21) sorbitan monooleate<br>polyoxyethylene (15) sorbitan dioleate | 1392.6<br>1392.7<br>1392.5<br>1392.7 |
| | 1788.9 | polyoxyethylene (36) sorbitan<br>polyoxyethylene (30) sorbitan monooleate<br>polyoxyethylene (24) sorbitan dioleate | 1789.2<br>1789.1<br>1789.2 |
| | 2362.0 | polyoxyethylene (43) sorbitan monooleate<br>polyoxyethylene (37) sorbitan dioleate | 2361.9<br>2362.0 |

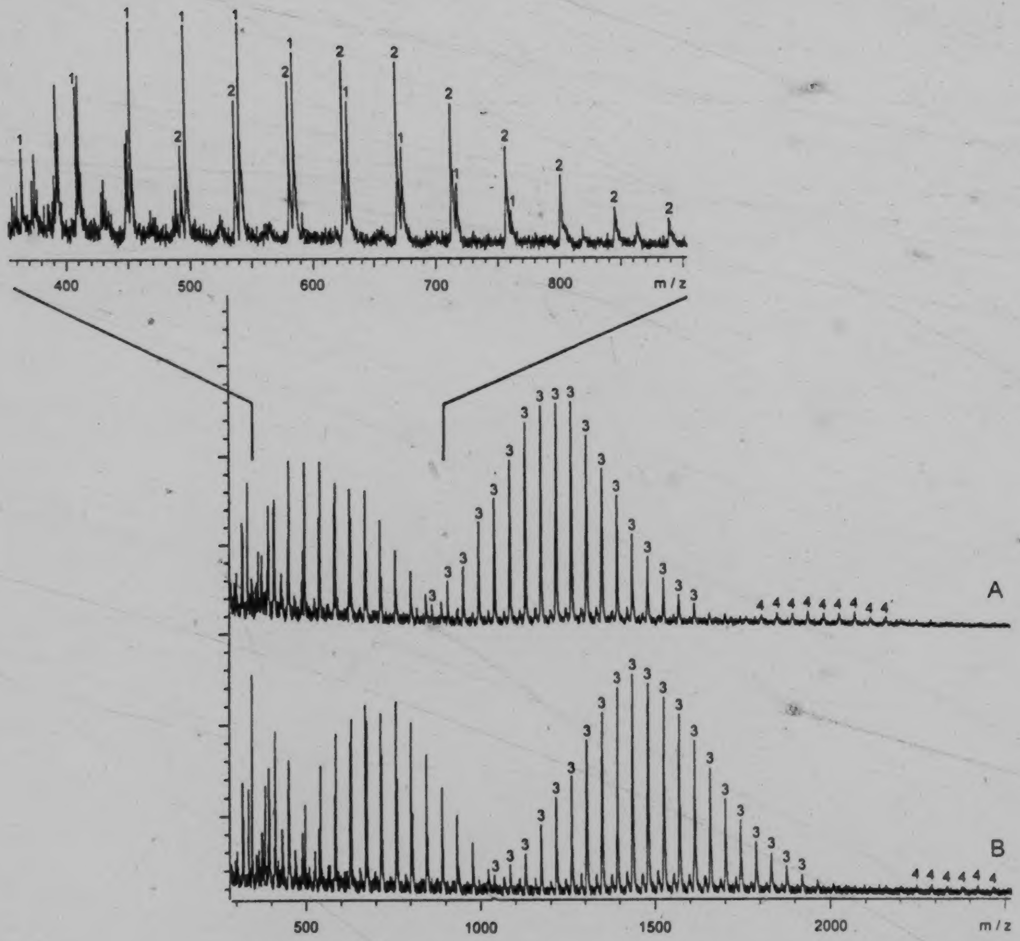


**Figure 4-4.** MALDI-TOF MS positive ion spectra of PS 60 (a) and PS 80 (b) after removal of the fatty acids by saponification. Concentrations were 5 mg/mL in aqueous 0.01 M potassium chloride. The peaks were identified as belonging to the following four series: 1 - polyoxyethylenes; 2 - isosorbide carbowaxes; 3 - sorbitan carbowaxes; 4 - disorbitan carbowaxes. The small peaks among series 3 are sodium adducts of the sorbitan carbowaxes. Unlabelled peaks below *m/z* 500 result from fragmentation of the matrix.

Although similar in shape, the mass distributions for the saponified PS 80 occur in a higher molecular weight range than do the saponified PS 60 mass distributions (Figure 4- 4). This phenomenon suggests that PS 80 exhibits a higher degree of ethylene oxide polymerization than does PS 60, which is notable given that these compounds are both defined to contain a molar average of 20 ethylene oxide residues. It is clear from these spectra that the average number of ethylene oxide residues is substantially higher in PS 80 than in PS 60, a situation which may contribute to differences in emulsification properties.

Interestingly, it appears that a significant proportion of the species present in both PS 60 and PS 80 do not contain a fatty acid at all, as revealed by the comparison of mass distributions before and after saponification, shown in Figure 4-5. Many of the molecular weights observed before saponification are identical to those appearing after

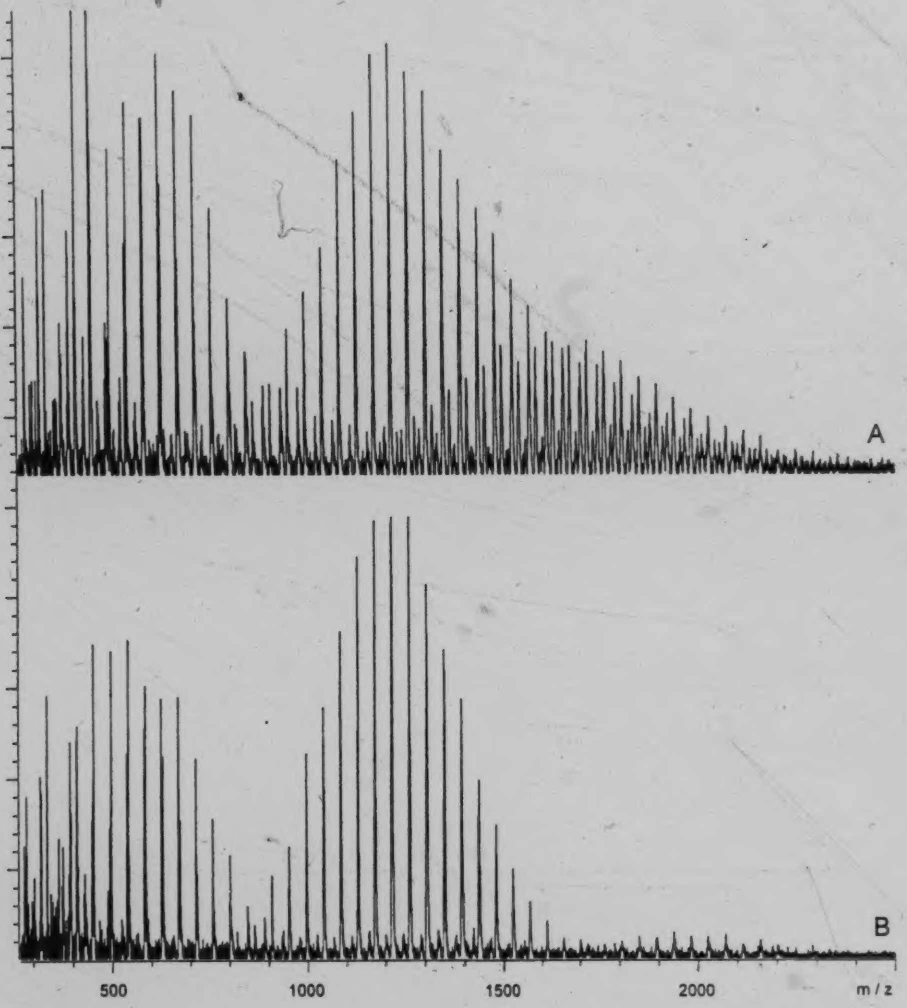


**Figure 4- 5.** Comparison of the MALDI-TOF MS positive ion spectrum of PS 60 before (a) and after (b) saponification.

saponification, implying the absence of esterified fatty acids in the original product. Lacking a hydrophobic region, these components would not be considered good emulsifiers.

MALDI-TOF MS analysis provides the most complete and detailed account to date of the molecular composition of polysorbate emulsifiers. It confirms the polydispersity of the ethylene oxide chains, and offers insight into the degree of esterification and the relative distribution of sorbitan- and sorbide-based species. For a more complete description of attributes such as stereochemistry of the constituents or the position of esterification, complementary analytical methods would be required, but MALDI-TOF MS is an excellent tool for providing a snapshot of polysorbate composition. The technique also demonstrates potential for future quantitation of the various polysorbate components, as well as polysorbate analysis in complex food matrices.

As a result of this analysis, it can be seen that the commercial names of polysorbate products are simplifications of the true nature of the products. The efficacy of polysorbate emulsifiers is probably due largely to their heterogeneity, which until now could only be estimated by statistical probabilities. MALDI-TOF MS offers a fast and simple method for characterizing their molecular complexity, with potential applications for quality control during manufacture. Furthermore, by gaining an understanding of polysorbate composition, more informed choices can be made when selecting polysorbates for both new and existing food formulations.

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# Chapter 5

## Identification and Quantification of Flavonol Glycosides in Almond Seedcoats Using MALDI-TOF Mass Spectrometry[1]

### INTRODUCTION

The search for dietary components which confer health benefits beyond those traditionally ascribed to the macro- and micro-nutrients is an emerging area of nutritional research. Particular attention has been directed toward identifying certain phytochemicals which may protect against the development of chronic diseases. Several epidemiological studies have linked nut consumption with reduced risk of coronary heart disease (Kushi et al., 1996; Hu et al., 1998; Sabate, 1999). Furthermore, nuts have been shown to favourably alter serum lipid levels whereby the magnitude of the effect is substantially greater than would be predicted by the fatty acid profile alone (Jenkins et al., 1997; Spiller et al., 1998; Kris-Etherton et al., 1999). This evidence suggests that the numerous bioactive constituents in nuts may contribute considerably to the overall health benefits. For this reason, the characterization and quantification of these compounds is an important first step toward determining their specific actions and efficacies.

Almonds are an attractive subject of study due to their widespread consumption and increasing popularity. Although published information on almond flavonoid composition is scarce, the presence of certain polyphenols has been confirmed. Several studies report procyanidins of varying degrees of polymerization occurring in the almond seedcoat, almond flesh, and almond fruit (Brieskorn and Betz, 1988; Plumb et al., 1998; de Pascual-Teresa et al., 1998; Lazarus et al., 1999; Catterall et al., 2000). Other flavonoids are known to occur naturally in all nuts (Rainey et al., 1997), but currently no characterization in almonds has been reported. As antioxidants, flavonoids help to quench free radicals and regenerate other antioxidants. Several *in vitro* studies have demonstrated the ability of flavonoids to inhibit the oxidation of low density lipoprotein (LDL) (Serafini et al., 2000), modulate platelet activation (Rein et al., 2000), and inhibit cancer cell proliferation (Kampa et al., 2000).

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[1] A version of this chapter has been published. Frison-Norrie, S.; Sporns, P. Identification and quantification of flavonol glycosides in almond seedcoats using MALDI-TOF MS. *J. Agric. Food. Chem.* **2002**, *50*, 2782-2787.

The importance of flavonol glycosides, a subclass of flavonoids, has led to the development of a number of methods for identification and quantification. Traditionally, flavonol glycosides have been characterized using paper chromatography, thin-layer chromatography, and UV spectroscopy (Harborne et al., 1975). Recently, the application of high performance liquid chromatography (HPLC) for their separation and quantification has become predominant (Harborne et al., 1988; Crozier et al., 1997). The coupling of HPLC to mass spectrometry methods such as electrospray, has been adopted to provide molecular weight information and to generate characteristic fragment ions for structural elucidation (Hakkinen et al., 1998; Mauri et al., 1999). However, the most powerful techniques for molecular structure determination of flavonol glycosides remain $^1$H and $^{13}$C NMR spectroscopy (Vilegas et al., 1999; Fico et al., 2000).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is new analytical technique first introduced in 1987 (Karas et al., 1987). Originally developed for the analysis of large biomolecules, MALDI-TOF MS has been successfully applied to a number of analytical problems in the food area, although its food applications are still limited (Sporns and Wang, 1998). The MALDI-TOF MS technique offers several advantages over other methodologies including ease of sample preparation, rapid generation of spectra, tolerance of impurities, and minimal fragmentation allowing direct access to molecular weight. However, isomers with the same molecular weight cannot be differentiated. Qualitative and quantitative analysis of anthocyanins, which are structurally similar to flavonols, has been reported by Wang and Sporns (1999). The first application of MALDI-TOF MS to study food flavonol glycosides appeared in 2000 (Wang and Sporns), but quantitative analysis was not addressed. The objectives of the current research were to develop and validate a MALDI-TOF MS methodology for qualitative and quantitative analysis of flavonol glycosides in almond seedcoats.

## MATERIALS AND METHODS

**Materials and Reagents.** A composite sample of almond seedcoats (*Prunus dulcis*), which had been removed from the almond meat by blanching, was supplied by the Almond Board of California (Modesto, CA). Rutin (quercetin-3-rutinoside) was purchased from Sigma Chemical Co. (St. Louis, MO). Isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside, kaempferol-3-rutinoside, and kaempferol-3-glucoside were obtained from Extrasynthese S.A. (Genay Cedex, France). The matrix 2',4',6'-trihydroxyacetophenone monohydrate was from Aldrich Chemical Co. (Milwaukee, WI). All water was double deionized (Milli-Q water purification system, Millipore Corp., Bedord, MA).

**Extraction and Isolation of Flavonol Glycosides.** A 5.0 g sample of almond seedcoats, ground to a coarse powder, was stirred with 100 mL of 70% methanol for 30 min., then filtered by gravity through Whatman No. 4 filter paper. Methanol was removed from the filtrate using a rotary evaporator with bath temperature of 35 $^{o}$C. The filtrate volume was adjusted to 25 mL with water. The methanol-free filtrate (5 mL) was loaded onto a Sep-Pak $C_{18}$ cartridge (Waters Corp., Milford, MA) which had been preconditioned with 5 mL methanol and 5 mL water. The cartridge was washed three times with 10 mL water and eluted with 2 mL of 70% methanol for samples analyzed by HPLC. Alternatively, flavonol glycosides were eluted with 2 mL of 0.01M NaCl in 70% methanol for MALDI-TOF MS analysis. The orange-brown extracts were refrigerated until use.

**MALDI-TOF MS.** MALDI-TOF MS analysis was performed using a Proflex III instrument with a linear flight tube (Bruker Analytical Systems Inc., Billerica, MA). A saturated solution of THAP in acetone (1 μL) was applied to the stainless steel probe and allowed to air-dry, followed by application of 1 μL of analyte solution. Analytes were ionized using a 3 ns nitrogen laser pulse (337 nm) and accelerated under 20 kV using delayed extraction before entering the time-of-flight mass spectrometer. Laser strength was adjusted to provide optimal signal-to-noise ratio and samples were analyzed in the positive ion mode.

**Determination of Rutin Linearity and Response Factors by MALDI-TOF MS.** Stock solutions of rutin, isorhamnetin-3-rutinoside, and kaempferol-3-rutinoside were prepared by dissolving each in 70% methanol at 1.00 mg/mL. A series of dilutions were prepared to give concentration ratios of rutin to either isorhamnetin-3-rutinoside or kaempferol-3-rutinoside as follows: 0.25, 0.5, 1.0, 1.5, 2.0. Each of these solutions was analyzed by MALDI-TOF MS and the peak height ratios (using the sum of all proton and sodium adduct peaks for both fragment ions and parent ions) were compared to the concentration ratios to obtain the response factors.

**Quantification using MALDI-TOF MS.** Quantification of flavonol glycosides was achieved using rutin as an internal standard. A 40 µL aliquot of rutin stock solution (1.00 mg/mL in 70% methanol) and 360 µL of 0.01M NaCl in 70% methanol were added to 600 µL of almond seedcoat eluent to give a final rutin concentration of 40 µg/mL. This solution was spotted on five separate positions on the MALDI probe. A single spectrum was then generated for four of these positions by randomly collecting 120 laser shots. The probe was then washed and spotted a second time at five positions, and four more spectra were generated for a total of 8 spectra. Peak heights for proton and sodium adducts were determined for all flavonol species from each spectrum.

**HPLC Analysis.** The HPLC system was composed of a Varian Vista 5500 pump (Varian Canada Inc., Mississauga, Ontario, Canada), a Varian 9090 autosampler, and a SpectroMonitor III UV detector (LDC/Milton Roy, Riviera Beach, FL). The system was fitted with a 75 x 4.5 mm i.d. preinjection $C_{18}$ saturator column containing silica-based packing (12 µm) and a 50 x 4.6 mm i.d. guard column containing Supelco LC-18 reversed phase packing, 20 to 40 µm, (Supelco, Bellefonte, PA). Flavonol glycosides were separated on a Supelcosil SPLC-DB-18 250 x 10 mm i.d. (5 µm) preparative reversed-phase column (Supelco, Bellefonte, PA). The solvent system consisted of HPLC-grade water (solvent A) and acetonitrile (solvent B). Flow rate was maintained at 5 mL/min, with a linear gradient of solvent A and the following proportions (v/v) of solvent B: 0 min, 15% B; 0-40 min, 15-18% B; 40-44 min, 18-31% B; 44-46 min, 31% B; 46-48 min, 31-15% B. Total run time was 55 min, and detection was at 354 nm. Injection volume of standard and sample solutions was 90 µL. A Shimadzu CLASS-VP chromatography data system (Shimadzu Scientific Instruments Inc., Columbia, MD) was used to monitor and to integrate the eluted peaks.

**Quantification using HPLC.** A standard curve was prepared using four concentrations of a standard mixture. The relative proportions of flavonol glycosides in the standard mixtures are similar to those found naturally in the almond seedcoat extract. A stock solution was prepared with isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside, kaempferol-3-rutinoside, kaempferol-3-glucoside, and rutin (internal standard) at concentrations of 120, 40, 20, 12, and 120 μg/mL, respectively, in 90% methanol. The stock solution was then diluted by a factor of 2, 4, and 8. Three replicate injections of almond seedcoat extract containing rutin as an internal standard were performed.

## RESULTS AND DISCUSSION

MALDI-TOF MS analysis is commonly used for molecular identification purposes, as molecular weights can be determined rapidly and accurately. More challenging is the application of this tool for quantification purposes, as several obstacles must be overcome. Difficulties in achieving reproducible homogeneous crystal beds can lead to inherent variability among identical preparations. Multiple analytes in solution can compete for ionization energy or exhibit variations in ionization potential. Multiple ion adducts may appear for single compounds, with different compounds displaying different preferences for particular ion forms. Finally, fragmentation, while diagnostic for identification purposes, can complicate the issue of quantification if fragment ions and analyte ions are identical.

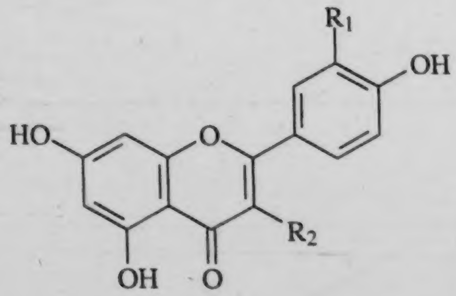
**Controlling Variability.** One factor which can affect the degree of variability among replicates is the uniformity of the matrix-analyte crystal bed. 2', 4', 6'-Trihydroxyacetophenone monohydrate was selected as a matrix because it has proven successful in our laboratory in the past for analyzing flavonol glycosides (Wang and Sporns, 2000), and has demonstrated good spot-to-spot repeatability and tolerance of impurities. When dissolved in acetone, the solution evaporates quickly to produce a lawn of small homogeneous crystals (Wang et al., 1999). However, the nature of the solvent system which is subsequently spotted on top of the matrix can affect the homogeneity of the final crystal formation. Solutions prepared in 90% methanol completely redissolved the matrix and caused very irregular crystals. Conversely, solutions prepared in 70% methanol only redissolved a small proportion of the matrix. The remaining undissolved matrix acted as seed crystals for the rapid recrystallization of analyte and matrix in a more regular fashion. Multiple analyses of standards in 90% methanol showed much higher standard deviations in relative responses than did standards dissolved in 70% methanol (data not shown). For this reason, all MALDI-TOF MS analyses were carried out using solutions prepared in 70% methanol.

As a further measure to reduce error, ten positions on the probe were spotted and one spectrum was generated at each position. Of these ten spectra, the eight best ones were chosen for further analysis. Spectrum selection criteria included high response, good resolution, good signal-to-noise ratio, and consistency in relative response with respect to the other spectra.

**Response Factors of Flavonol Glycosides in MALDI-TOF MS.** Despite large variations in absolute response, relative responses remained relatively constant across many trials. On this basis, the relative response of analytes to an internal standard can be used for quantification purposes. Rutin was chosen as an internal standard for almond seedcoat analysis because of its absence in the sample and its structural similarity to the analytes (Table 5-1). However, due to inherent differences in crystallization and ionizability, chemically similar compounds may exhibit different responses at the same concentration. Thus, it is important to determine the analyte response factors prior to quantification.

The response of rutin was linear with standards of isorhamnetin-3-rutinoside and kaempferol-3-rutinoside ($R^2$=0.99 for both) and response factors were determined to be 0.5127 and 0.8481, respectively. In other words, at the same concentration, the response of rutin will be roughly 51% as intense as isorhamnetin-3-rutinoside and 85% as intense as kaempferol-3-rutinoside. The only structural differences among these molecules are in the aglycone (Table 5-1). Since the rutinoside analogues share an identical aglycone with their glucoside analogues, as well as fragment to produce them, these response factors were assumed to be valid for the respective glucosides as well.

**Table 5-1.** Chemical structure of flavonol glycosides.



| Flavonol | $R_1$ | $R_2$ |
|---|---|---|
| kaempferol | H | OH |
| quercetin | OH | OH |
| isorhamnetin | OCH3 | OH |
| kaempferol-3-glucoside | H | *O*-glucose |
| quercetin-3-glucoside | OH | *O*-glucose |
| isorhamnetin-3-glucoside | OCH3 | *O*-glucose |
| kaempferol-3-rutinoside | H | *O*-rutinose |
| quercetin-3-rutinoside (rutin) | OH | *O*-rutinose |
| isorhamnetin-3-rutinoside | OCH3 | *O*-rutinose |

**Ionization Patterns of Flavonol Glycosides.** Quantification using MALDI-TOF MS can be further complicated by multiple ionization. Peak heights of all flavonol glycosides in the sample (including internal standard peaks) were calculated using the software. Based on molecular weight, four flavonols were identified in the almond seedcoat extract as isorhamnetin rutinoside, isorhamnetin glucoside, kaempferol rutinoside, and kaempferol glucoside (Table 5-2). Excess sodium chloride was added to the almond extracts to suppress the formation of potassium adduct ions. Under these conditions, all three types of almond seedcoat flavonols (isorhamnetin, kaempferol, and quercetin derivatives) exhibited similar ionization patterns. Figure 5-1 shows that $[M+H]^+$ adducts were produced for aglycone peaks, $[M+H]^+$ and $[M+Na]^+$ adducts for

**Table 5-2.** Identification of flavonol glycoside peaks in mass spectrum of almond seedcoat extract.

| Flavonol Glycoside | Exact Mass | MALDI-TOF MS Mass | Observed Adduct Ions |
|---|---|---|---|
| kaempferol | 287.26 | 286.57 | $[M+H]^+$ |
| isorhamnetin | 317.29 | 316.69 | $[M+H]^+$ |
| kaempferol glucoside | 449.42 | 449.07 | $[M+H]^+$ |
| | 471.40 | 471.09 | $[M+Na]^+$ |
| isorhamnetin glucoside | 479.45 | 479.17 | $[M+H]^+$ |
| | 501.43 | 501.16 | $[M+Na]^+$ |
| kaempferol rutinoside | 595.58 | 595.34 | $[M+H]^+$ |
| | 617.56 | 617.41 | $[M+Na]^+$ |
| isorhamnetin rutinoside | 625.61 | 625.46 | $[M+H]^+$ |
| | 647.59 | 647.46 | $[M+Na]^+$ |
| | 669.58 | 669.48 | $[M+2Na-H]^+$ |

glucoside peaks, and $[M+H]^+$, $[M+Na]^+$, and $[M+2Na-H]^+$ adducts for rutinoside peaks, for a total of six peaks per type of flavonol. Kaempferol rutinoside, a minor compound, did not express the double sodium minus proton adduct. It appears that as the carbohydrate component increases, preference for the sodium ion form also increases. The total analyte response was taken to be the total of all associated adduct peaks. Therefore, peak heights of the two glucoside adduct peaks and the three rutinoside adduct peaks were summed to give the total response for the respective compounds.

**Fragmentation of Flavonol Glycosides.** Although diagnostic for identification purposes, fragmentation can cause considerable difficulty for quantification. As shown in Figure 5-2, in-source fragmentation of rutinoside standards to yield both glucoside and aglycone ions can be responsible for a substantial proportion of the response in MALDI-TOF MS. The characteristic peaks at $[M-146]^+$, indicated the loss of the labile rhamnoside residue, resulting in the glucoside. Subsequent loss of another 162 mass

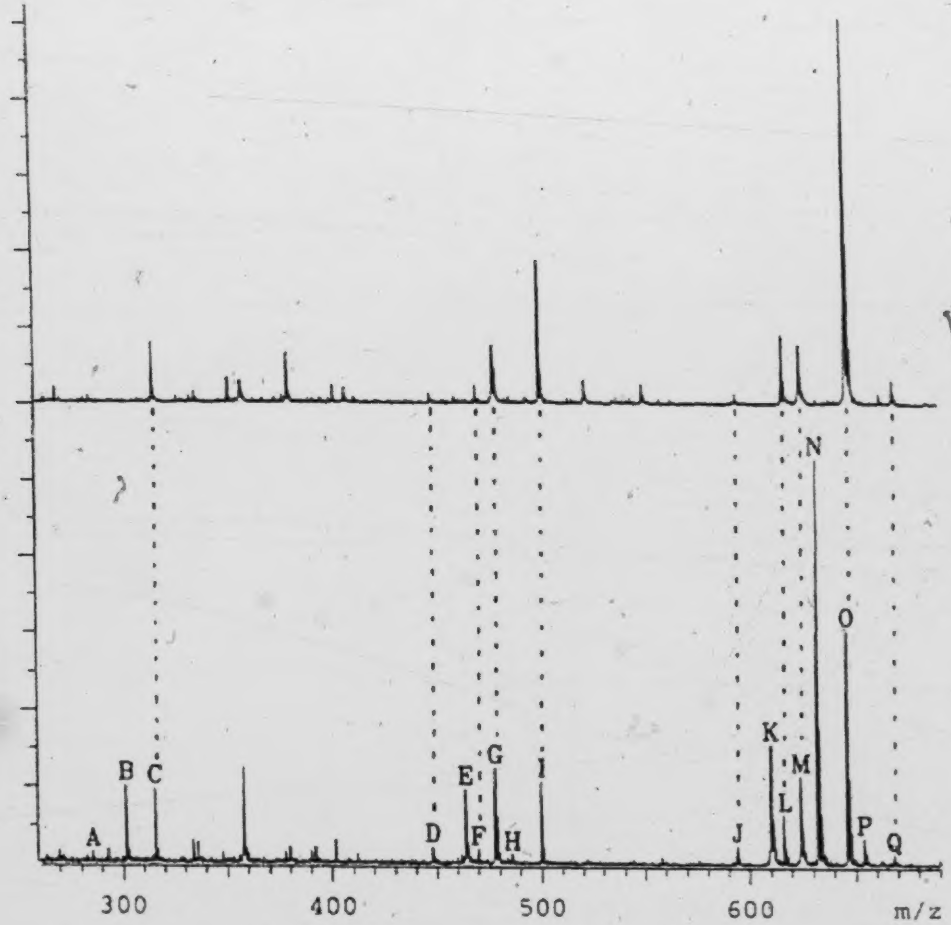


**Figure 5-1.** MALDI-TOF MS positive ion spectra of almond seedcoat extract with added sodium chloride. Top: almond seedcoat extract. Bottom: almond seedcoat extract with rutin added as internal standard. Spectra are the sum of 120 laser pulses. Internal standard peaks are indicated in bold. A: [kaempferol + H]$^+$, **B: [quercetin + H]$^+$**, C: [isorhamnetin + H]$^+$, D: [kaempferol glucoside + H]$^+$, **E: [quercetin-3-glucoside + H]$^+$**, F: [kaempferol glucoside + Na]$^+$, G: [isorhamnetin glucoside + H]$^+$, **H: [quercetin-3-glucoside + Na]$^+$**, I: [isorhamnetin glucoside + Na]$^+$, J: [kaempferol rutinoside + H]$^+$, **K: [quercetin-3-rutinoside + H]$^+$**, L: [kaempferol rutinoside + Na]$^+$, M: [isorhamnetin rutinoside + H]$^+$, **N: [quercetin-3-rutinoside + Na]$^+$**, O: [isorhamnetin-3-rutinoside + Na]$^+$, **P: [quercetin-3-rutinoside + 2Na - H]$^+$**, Q: [isorhamnetin rutinoside + 2Na - H]$^+$. Unlabelled peaks are matrix fragments.

units, equivalent to a glucose residue, resulted in the aglycone peak. Although aglycone peaks were observed in the almond mass spectrum, it is assumed that these arose entirely from fragmentation of the parent rutinoside and glucoside ions, since HPLC data did not indicate aglycones in the sample. However, chromatography data did confirm the presence of glucosides in the sample. Therefore, the observed glucoside peak response in the mass spectra was due to a combination of glucoside present originally in the sample, and that resulting from fragmentation of the rutinoside. In contrast, all of the aglycone response was due to fragmentation. As illustrated in Figure 5-3, one can construct a picture of the original concentration of flavonol glycosides in the almond sample by determining the proportion of fragment ion response attributable to each parent ion.

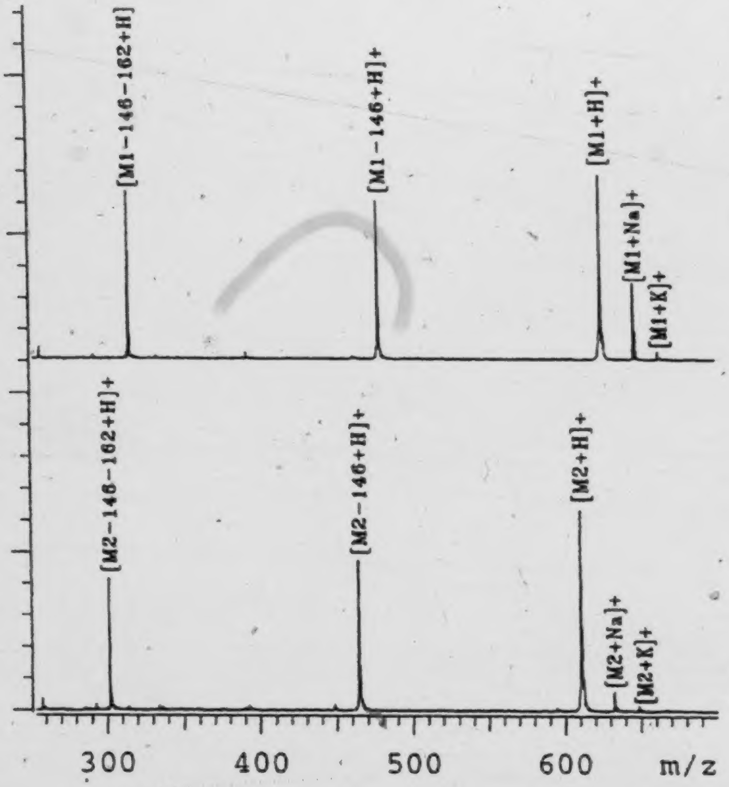


**Figure 5-2.** MALDI-TOF MS natural cation spectra of flavonol glycoside standards. Top: M1 = isorhamnetin-3-rutinoside ($1.5 \times 10^{-3}$M in 70% methanol). Bottom: M2 = rutin ($1.5 \times 10^{-3}$M in 70% methanol).

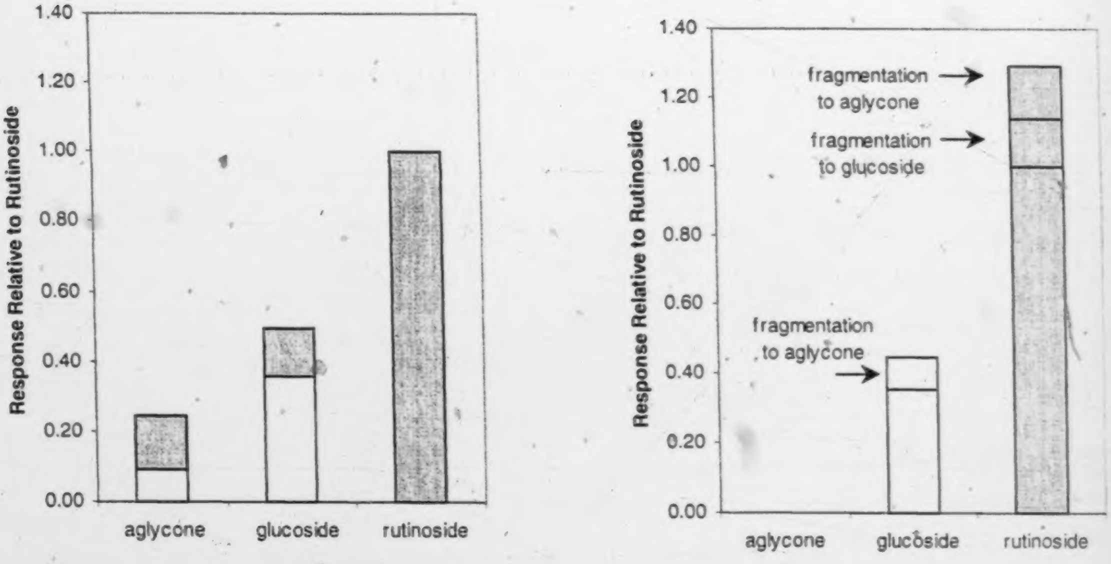


**Figure 5-3.** Schematic representation of the method used to correct for fragmentation of the rutinoside and glucoside analytes in MALDI-TOF MS analysis. ■ represents proportion of response from rutinoside, □ represents proportion of response from glucoside. a) Relative responses observed in a typical spectrum for aglycone, glucoside, and rutinoside ions. b) Relative responses of these ions after correcting for fragmentation.

A comparison of fragmentation ratios (peak height ratio of fragment ions to parent ions) between rutin and isorhamnetin-3-rutinoside showed considerable variation in the absolute value as indicated by large standard deviations (Table 5-3). However, within any single MALDI-TOF MS run, the relative fragmentation ratios of these two flavonol rutinosides were similar. Previous work by Wang and Sporns (2000) also reported similar fragmentation among all flavonol glycosides using MALDI-TOF MS. For this reason, it was concluded that the fragmentation pattern of the rutin internal standard was predictive of the fragmentation patterns of rutinosides present in the sample. Using the internal standard fragmentation pattern as a model, the proportion of glucoside and aglycone response due to rutinoside fragmentation was calculated for each replicate. The remaining proportions of glucoside and aglycone responses were therefore representative of the glucoside concentration. The proportion of parent glucoside and rutinoside ions which

**Table 5-3.** Ratios of fragment ions to parent ions for mixtures of rutin and isorhamnetin-3-rutinoside standards.[a]

| | **isorhamnetin-3-rutinoside** | **rutin** | **isorhamnetin-3-rutinoside/rutin** |
|---|---|---|---|
| **[M-146]**[b] | 0.52 (0.12) | 0.54 (0.12) | 0.96 (0.07) |
| **[M-146-162]**[c] | 0.86 (0.36) | 0.83 (0.35) | 1.04 (0.08) |
| **Total (fragment ions[d]/parent ions[e])** | 1.40 (0.52) | 1.37 (0.51) | 1.02 (0.07) |

[a] All data are means of 21 replicates, and numbers in parentheses represent the standard deviations. Flavonol glycoside standards were prepared by mixing appropriate amounts (v/v) from 1.00 mg/mL stock solutions in 70% methanol to give the following concentration ratios of rutin to isorhamnetin-3-rutinoside: 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, 5.0. Three replicate spectra were obtained at each concentration. [b] Proton and sodium adduct ions from loss of rhamnose residue. [c] Proton adduct ions from loss of rutinose residue. [d] Total of proton and sodium adduct ions from loss of either one or both carbohydrate residues. [e] Total of unfragmented proton and sodium adduct molecular ions.

appear in the mass spectrum as fragment ions were accounted for by a fragmentation correction factor.

The analyte concentration (in µg/g, fresh weight basis) was calculated according to Eq. 1. (where "peak height rutin" is the sum of all proton and sodium adduct peak heights associated with rutin and its fragment ions). See also Appendix I.

**Eq.1.** [analyte] = [rutin] x (peak height analyte / peak height rutin) x dilution factor x response factor x fragmentation correction factor

**Comparison of MALDI-TOF MS and HPLC Results.** HPLC is currently the most common method for analyzing flavonol glycosides, both qualitatively and quantitatively. For this reason, an HPLC protocol was chosen to compare with the MALDI-TOF MS results, in order to evaluate the effectiveness of this mass spectrometric method, and to validate the assumptions made. Further evidence for the identity of the four compounds of interest in almond seedcoat was provided by peak retention times similar to those of pure standards. Table 5-4 shows the results of flavonol glycoside analysis in almond seedcoats using both MALDI-TOF MS and HPLC methods. The HPLC calculations were based on a linear relationship and response factors when compared with rutin of 0.4460, 0.3172, 0.8344, and 0.5491 for isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside, kaempferol-3-rutinoside, and kaempferol-3-glucoside, respectively, at 354 nm. The different response factors for compounds with the same aglycone likely arose because of variation in the solvent. Thus, for HPLC quantification purposes, the need for standards is evident, in that response factors will differ slightly for each variation in solvent conditions.

**Table 5-4.** Comparison of MALDI-TOF MS and HPLC determination of flavonol glycoside concentration in almond seedcoat (μg/g) [a].

| | MALDI-TOF MS [b] | HPLC [c] |
|---|---|---|
| isorhamnetin rutinoside | 51 (5) | 53 (3) |
| isorhamnetin glucoside | 18 (3) | 13 (1) |
| kaempferol rutinoside | 18 (3) | 22 (1) |
| kaempferol glucoside | 6 (1) | 7 (1) |
| Total | 93 (12) | 95 (6) |

[a] Concentration determined on a fresh weight basis. [b] Numbers in parentheses represent standard deviations where n=8. [c] Numbers in parentheses represent standard deviations where n=3.

The results for individual flavonol glycosides and the total, show good correlation between the two analytical methods. The HPLC method may have slightly overestimated the isorhamnetin rutinoside concentration and slightly underestimated the isorhamnetin glucoside concentration, as these two peaks were not completely resolved (Figure 5-4). No modifiers such as TFA were added, due to the possibility of hydrolyzing the rhamnose residue. Of the two methods, MALDI-TOF MS exhibited a higher variability. However, MALDI-TOF MS is much faster than HPLC, requiring about 20 min to determine peak heights for 8 probe positions, whereas one HPLC analysis lasted nearly an hour. Another advantage of MALDI-TOF MS is the ability to identify peaks based on mass, rather than on comparison with retention times of standards, which may be unavailable, as for HPLC.

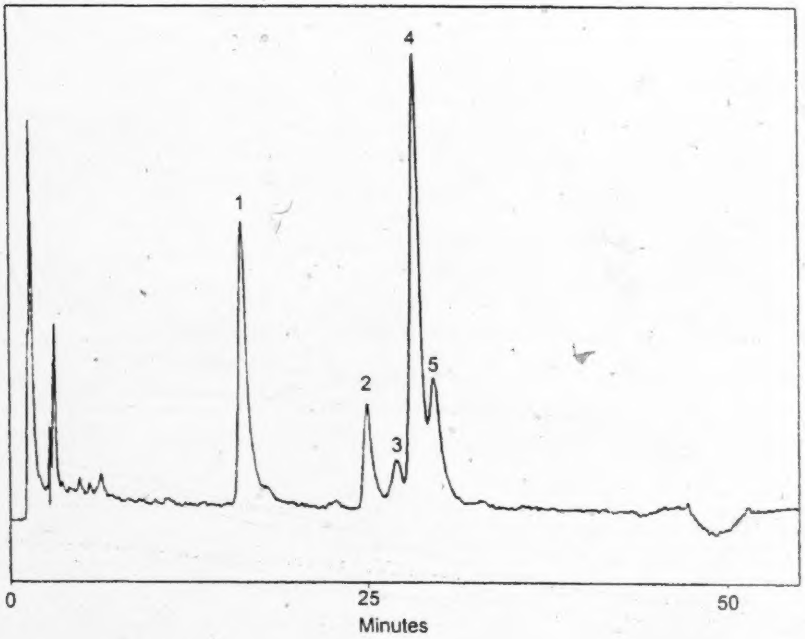


**Figure 5-4.** HPLC chromatogram of almond seedcoat extract with added rutin. (1) rutin, (2) kaempferol rutinoside, (3) kaempferol glucoside, (4) isorhamnetin rutinoside, (5) isorhamnetin glucoside.

Carbohydrate moieties can bind to various positions on the parent flavonoid, but the MALDI-TOF MS technique is unable to distinguish among these isoforms. Characteristic fragmentation provides some structural information, but to achieve more detailed information would require further analysis using other techniques such as nuclear magnetic resonance (NMR). However, it is postulated that the carbohydrates are attached at the 3-position, as this form is the most common (Hollman and Arts, 2000), and because chromatography data showed similar retention times between flavonol-3-glycoside standards and the unknown compounds in the sample.

Because there was a high correlation between the results of the mass spectrometric and chromatographic quantitative analyses, the assumptions made for MALDI-TOF MS quantification are valid in this system. Therefore, MALDI-TOF MS is an excellent complement to conventional analytical methods. As such, there is potential for MALDI-TOF MS to be used as a rapid and definitive screening tool for a wide variety of almond samples for the purposes of exploring varietal differences and determining authenticity.

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# Chapter 6

## Variation in the Flavonol Glycoside Composition of Almond Seedcoats as Determined by MALDI-TOF Mass Spectrometry[1]

### INTRODUCTION

Almonds are the seeds of varieties of *Prunus amygdalus*, a member of the Rosaceae family, which also includes apples, pears, peaches, and plums. The flesh of the seed is encased in a brown leathery coating, called the seedcoat, which protects the almond from oxidation and microbial contamination. Many food applications of almonds in bakery and confectionary items, cereals, snack formulations, and marzipan, require the flesh of the almond alone without the seedcoat. During the process of blanching, moist heat is used to loosen the seedcoat from the almond flesh, followed by gentle agitation to complete the separation.

Almond seedcoats are usually discarded after they are removed by blanching. Yet, recent investigations into the phytochemical composition of almonds have shown that the seedcoats may contain many potentially beneficial compounds, opening up new possibilities for the value of almond seedcoats. Studies by Brieskorn and Betz (1987) revealed that almond seedcoats are a rich source of sterols. Some types of sterols have been shown to reduce serum cholesterol levels (Wong, 2001). Takeoka et al. (2000) described three triterpenoids, betulinic acid, oleanoic acid, and ursolic acid, which have reported anti-inflammatory (Singh et al., 1994), anti-HIV (Kashiwada et al., 1998), and anti-cancer activities (Pisha et al., 1995).

Oxidative stress has been linked to the aging process, and to the development of arteriosclerosis and cancer. One potential way to combat oxidative stress is to consume a variety of antioxidants in the diet. The primary source of naturally-occurring dietary antioxidants is plant foods. Commonly found in edible plants are phenolic compounds which are thought to exhibit antioxidant activity through scavenging free radicals (Pietta, 2000). Knowledge about the absorption, bioavailability, and metabolism of dietary phenolics is also important to fully evaluate their potential health benefits.

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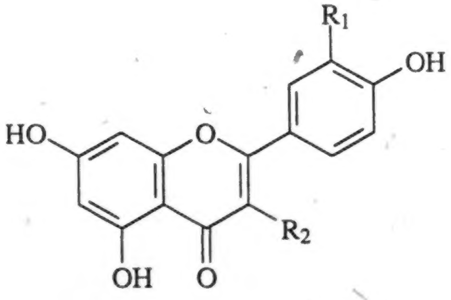
[1] A version of this chapter has been published. Frison, S.; Sporns, P. Variation in the flavonol glycoside composition of almond seedcoats as determined by MALDI-TOF mass spectrometry. *J. Agric. Food Chem.* **2002**, *50*, 6818-6822.

Sang et al. (2002a, 2002b) have isolated and characterized several important antioxidant phenolic compounds from almond seedcoats. Flavonoids are a subclass of phenolic compounds, which can be further classified into several structural subcategories, including proanthocyanidins and flavonol glycosides. Proanthocyanidins of varying degrees of polymerization have been identified in the almond seedcoat, almond flesh, and almond fruit (Brieskorn and Betz, 1988; de Pascual-Teresa et al., 1998; Plumb et al., 1998; Lazarus et al., 1999; Catterall et al., 2000) and are likely responsible for the reddish-brown hue of the seedcoat.

Our previous work has identified four flavonol glycosides in almond seedcoats: isorhamnetin rutinoside, isorhamnetin glucoside, kaempferol rutinoside, and kaempferol glucoside (Figure 6-1) using a technique known as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Frison-Norrie and Sporns, 2002, Chapter 5). We demonstrated the potential of this instrument to rapidly analyze flavonol glycosides in a complex extract. Also, we were able to reliably quantify these four analytes using an internal standard and correcting for in-source fragmentation. These findings prompted us to investigate the differences in flavonol glycoside composition across many varieties of almond seedcoat, using MALDI-TOF MS as a screening tool. In this paper we describe the variation in the seedcoat flavonol glycoside composition among sixteen varieties of almonds using MALDI-TOF MS.

## MATERIALS AND METHODS

**Materials and Reagents.** Seedcoats from sixteen varieties of almonds (*Prunus amygdalus*) were supplied by the Almond Board of California (Modesto, CA). The varieties were Aldrich, Butte, Carmel, Fritz, Le Grand, Mission, Monterey, Nonpareil, Padre, Peerless, Price, Ruby, Sauret, Sonora, Thompson, and Wood Colony. Isorhamnetin-3-rutinoside was obtained from Extrasynthese S.A. (Genay Cedex, France), and rutin (quercetin-3-rutinoside) was purchased from Sigma Chemical Co. (St. Louis, MO). The matrix 2',4',6'-trihydroxyacetophenone monohydrate was obtained from Aldrich Chemical Co. (Milwaukee, WI). All water was deionized by a Milli-Q water purification system (Millipore Corp., Bedford, MA).



| Flavonol | $R_1$ | $R_2$ |
|---|---|---|
| kaempferol | H | OH |
| quercetin | OH | OH |
| isorhamnetin | OCH3 | OH |
| kaempferol-3-glucoside | H | *O*-glucose |
| quercetin-3-glucoside | OH | *O*-glucose |
| isorhamnetin-3-glucoside | OCH3 | *O*-glucose |
| kaempferol-3-rutinoside | H | *O*-rutinose |
| quercetin-3-rutinoside (rutin) | OH | *O*-rutinose |
| isorhamnetin-3-rutinoside | OCH3 | *O*-rutinose |

**Figure 6-1.** Chemical structure of flavonol glycosides.

**Extraction of Flavonol Glycosides.** Extraction conditions were a modification of our earlier procedure (Chapter 5, Frison-Norrie and Sporns, 2002). Each variety of almond seedcoat was ground to a fine powder in a blender and sieved through a 20 mesh screen. An internal standard, rutin (500 μL of a 1 mg/mL solution in 90% methanol), was added to 5.0 g samples of each almond variety. The seedcoats were extracted twice by stirring each time with 50 mL of 70% methanol for 30 min. at room temperature, and the extracts were combined. Methanol was removed under reduced pressure, and the volume of the aqueous residue was adjusted to 25 mL with water. A Sep-Pak $C_{18}$ cartridge was preconditioned with 5 mL of methanol followed by 5 mL of water. Each extract was filtered through a 0.22 μm membrane and 5 mL was loaded onto a cartridge. The cartridges were washed with 30 mL of water and eluted with 2 mL of 0.01M NaCl in 70% methanol. The reddish-brown extract was refrigerated until analysis.

**MALDI-TOF MS.** MALDI-TOF MS analyses were performed using a Proflex III instrument with a linear flight tube (Bruker Analytical Systems Inc., Billerica, MA). A solution (20 mg/mL) of 2',4',6'-trihydroxyacetophenone monohydrate in acetone (0.7 μL) was applied to the stainless steel probe and allowed to air-dry, followed by application of 0.7 μL of analyte solution. Analytes were ionized using a 3 ns nitrogen laser pulse (337 nm) and accelerated under 20 kV using delayed extraction before entering the time-of-flight mass spectrometer. Laser strength was adjusted to provide optimal signal-to-noise ratios and samples were analyzed in the positive ion mode.

**Quantification of Flavonol Glycosides.** Three spectra of 80 laser shots each were obtained for each almond sample, and the results were averaged. Peak heights of the proton and sodium adduct ions for all flavonol species were determined relative to rutin and reported as μg of flavonol per g of seedcoat. Linearity ($R^2 = 0.99$), response ratios (0.5127 for isorhamnetin; 0.8481 for kaempferol) and quantification calculations were determined as described in Chapter 5.

## RESULTS AND DISCUSSION

Minor modifications were made to the sample preparation method reported in Chapter 5 for the purpose of improving the extraction efficiency and the consistency in conditions across the analysis of many samples. The particle size of the ground almond seedcoats was limited to that which could pass through a 20 mesh screen. Two 30 min. extractions of 50 mL of 70% methanol were used instead of one 30 min. extraction of 100 mL of 70% methanol. The internal standard, rutin, was added prior to extraction instead of prior to the column clean-up step to account for losses during work-up.

The ionic environment during MALDI-TOF MS ionization has the potential to alter the analyte response ratios, depending on the types of ions that are preferentially formed. To test the possibility that response ratios are dependent on the types of cations present, the relative responses of two standards (rutin and isorhamnetin-3-rutinoside) were studied under three conditions: in the presence of no added cations, in the presence of excess sodium ions (0.01 M), and in the presence of excess potassium ions (0.01 M) (Figure 6-2). Excess sodium chloride completely suppressed the formation of potassium adducts and created $[M+2Na-H]^+$ ions. Excess potassium chloride had the same effect, substituting potassium for sodium. In both cases, the proton adducts were unaffected. Proton, sodium, and potassium adducts of the rutinosides all appeared in the spectrum

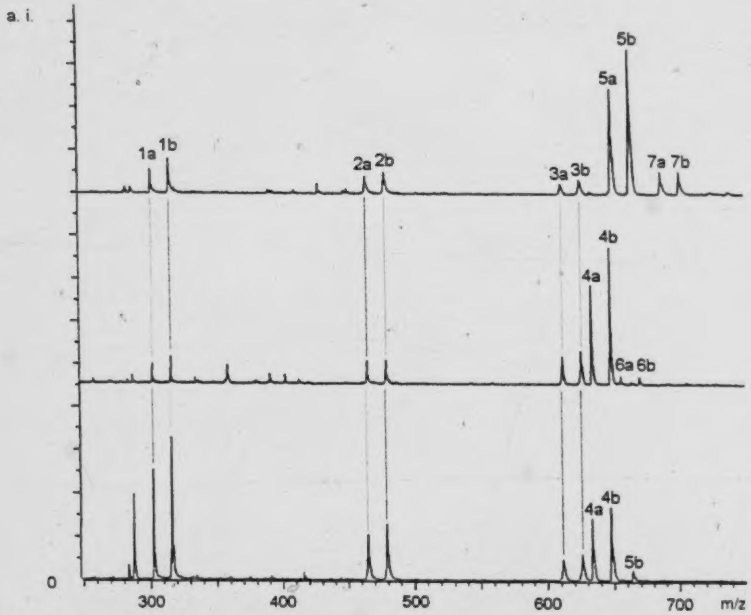


**Figure 6-2.** MALDI-TOF MS positive ion spectra of a mixture of rutin and isorhamnetin-3-rutinoside standards in a 1:1 concentration ratio (each at 0.5 mg/mL in 90% methanol). Bottom: Solution prepared with no added cations. Middle: Solution prepared with NaCl at a concentration of 0.01M. Top: Solution prepared with KCl at a concentration of 0.01M. (1a) [quercetin + H]$^+$, (1b) [isorhamnetin + H]$^+$, (2a) [quercetin-3-glucoside + H]$^+$, (2b) [isorhamnetin-3-glucoside + H]$^+$, (3a) [quercetin-3-rutinoside + H]$^+$, (3b) [isorhamnetin-3-rutinoside + H]$^+$, (4a) [quercetin-3-rutinoside + Na]$^+$, (4b) [isorhamnetin-3-rutinoside + Na]$^+$, (5a) [quercetin-3-rutinoside + K]$^+$, (5b) [isorhamnetin-3-rutinoside + K]$^+$, (6a) [quercetin-3-rutinoside + 2Na – H]$^+$, (6b) [isorhamnetin-3-rutinoside + 2Na – H]$^+$, (7a) [quercetin-3-rutinoside + 2K - H]$^+$, (7b) [isorhamnetin-3-rutinoside + 2K – H]$^+$.

with no added cations. Only the rutinosides showed a preference for alkali metal adducts. In this particular case, the fragment ions (glucosides and aglycones) appeared only as protonated species, although glucosides are also known to form sodium adducts (Chapter 5).

Table 6-1 shows the response ratios for isorhamnetin-3-rutinoside and its fragment ions as compared to the total rutin response. The relative responses given in the table represent the averages of three different MALDI-TOF MS analyses for each set of conditions. Sodium and potassium ions seem to be equally preferred for ionization, although sodium ions produce slightly lower variability on an individual peak basis. In the absence of added cations, the rutinoside response is de-emphasized, which consequently distorts the relative abundance of the fragment ions. Conversely, because the rutinosides have an affinity for alkali metal adducts, their response is accentuated in the presence of either sodium or potassium ions, again affecting the relative abundance of the fragment ions. At the same time, however, addition of sodium or potassium ions

**Table 6-1.** Comparison of response ratios of isorhamnetin-3-rutinoside and rutin standards under different ionic conditions.

| **Response Ratios**[a] | **No cations added** | **$Na^+$ added** | **$K^+$ added** |
|---|---|---|---|
| **isorhamnetin**[b] **/ rutin total**[c] | 0.47 +/- 0.13 | 0.15 +/- 0.01 | 0.16 +/- 0.03 |
| **isorhamnetin-3-glucoside**[b] **/ rutin total**[c] | 0.24 +/- 0.05 | 0.16 +/- 0.02 | 0.09 +/- 0.03 |
| **isorhamnetin-3-rutinoside / rutin total**[c] | 0.55 +/- 0.12 | 1.00 +/- 0.08 | 1.05 +/- 0.10 |
| **isorhamnetin-3-rutinoside total**[d] **/ rutin total**[c] | 1.27 +/- 0.04 | 1.31 +/- 0.11 | 1.29 +/- 0.07 |

[a] Values are the averages of three spectra.

[b] Isorhamnetin and isorhamnetin-3-glucoside are fragment ions of isorhamnetin-3-rutinoside

[c] Refers to the sum of the peak heights of all of the ions associated with rutin and its fragment ions.

[d] Refers to the sum of the peak heights of all of the ions associated with isorhamnetin-3-rutinoside and its fragment ions.

offers an advantage by significantly decreasing the spectrum-to-spectrum variability in the responses of the parent and fragment ions of the analyte (isorhamnetin-3-rutinoside) with respect to the response of the internal standard (rutin). Alternatively, it is possible that the presence of sodium or potassium ions suppresses fragmentation.

The ratio of the total isorhamnetin-3-rutinoside response ($H^+$ and $Na^+$ adduct ions of rutinoside plus fragment ions) to the total rutin response remains similar across all three conditions, implying that only the relative responses of individual peaks are affected by differences in the cation environment. The total rutin response remains constant despite changes in the relative ratios of individual ion peaks. Thus, to approximate the true flavonol composition in the almond seedcoat extracts by the mass spectrum, a correction factor is necessary to account for changes in the apparent abundance of fragment ions. As discussed in our previous work (Chapter 5), the fragmentation pattern of rutin can be used to predict the fragmentation pattern of the flavonol analytes, which accounts for this variability in response.

Figure 6-3 shows the variation in total flavonol glycoside content and the abundance of isorhamnetin rutinoside, isorhamnetin glucoside, kaempferol rutinoside, and kaempferol glucoside in the seedcoats of sixteen varieties of almonds. Only these four flavonol glycosides were detected in all of the samples tested, and isorhamnetin rutinoside was consistently the major species. The results for each almond variety represent the average of three MALDI-TOF MS analyses. The relative ratios of the individual flavonol glycoside peaks were very consistent; the average standard deviation among triplicate analyses for all samples was 9%. Quantification of flavonol glycosides was achieved according to the following equation:

**Eq.1.** [analyte] = [rutin] x (peak height analyte / peak height rutin) x dilution factor x response factor x fragmentation correction factor

For a detailed discussion of the quantification method, which is quite complex due to differential responses and fragmentation patterns, refer to Chapter 5. Within the varieties studied, the total flavonol glycoside content ranged from 75 μg/g (per gram of almond seedcoat) to nearly 250 μg/g. Further study is needed to determine to what extent

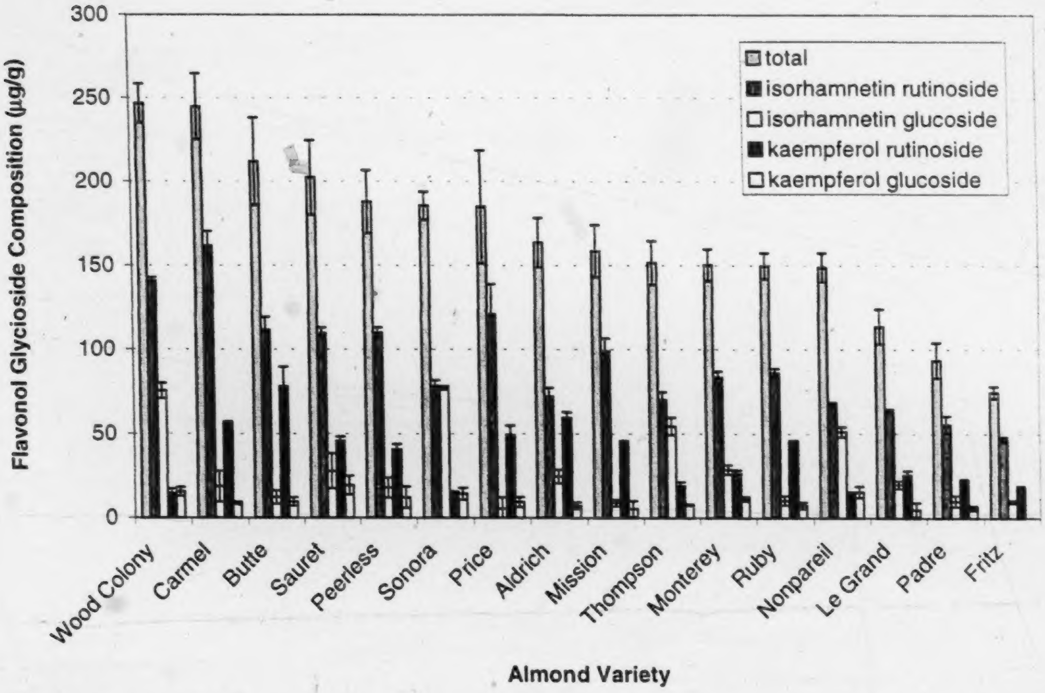


**Figure 6-3.** Total flavonol glycoside content and individual flavonol glycoside composition of sixteen almond varieties. Results for each variety are averages based on three MALDI-TOF MS spectra.

the variation in composition reflects inherent varietal differences as opposed to differences in growing conditions, handling, processing, and storage.

In a recent study by Sang et al. (2002a), nine antioxidative phenolic compounds were isolated from almond seedcoats: four flavonol glycosides, one flavanone glycoside, and three benzoic acid derivatives. In keeping with our results, they confirmed the presence of isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside, and kaempferol-3-rutinoside. However, their work did not report kaempferol-3-glucoside, which we had previously detected by MALDI-TOF MS and also identified by its high-performance liquid chromatography (HPLC) retention time (Chapter 5). Instead, they reported isorhamnetin-3-galactoside. We could not have differentiated this compound from isorhamnetin-3-glucoside by MALDI-TOF MS since these two compounds have identical molecular weights. Furthermore, the resolution we had previously achieved using HPLC probably would not have been sufficient to resolve the two isomers. Sang et al. (2002a) performed an extensive chromatographic separation where isolation of the galactoside from the glucoside could have been achievable. Therefore, it is possible that both of these isomers exist in almond seedcoats, but that our methodology cannot differentiate them.

The flavanone glycoside reported by Sang et al. (2002a), naringenin-7-glucoside (prunin), is chemically similar to the flavonol glycosides, but has a unique molecular weight. Also, according to their yield data, prunin is present in amounts comparable to the flavonol glycosides. In spite of these features, it was not detected by our MALDI-TOF MS protocol.

The simple extraction with 70% methanol and crude purification on a Sep-Pak $C_{18}$ cartridge is not selective for flavonol glycosides. The reddish-brown colour of the extract is a testament to the presence of other compounds, as flavonol glycosides themselves are yellow or nearly colourless. When considering the number of phytochemicals that have been identified in almond seedcoats (terpenoids, sterols, proanthocyanidins, benzoic acid derivatives, prunin, and flavonol glycosides) it is obvious that many compounds present in the extract are not detectable by our MALDI-TOF MS method. Differences in ionizability may play a role. Depending on their chemical nature and the sample preparation conditions, some families of compounds ionize preferentially, suppressing the ion formation of other groups of compounds. Only those which ionize preferentially will be detected. This feature partially explains the relative tolerance to impurities demonstrated by MALDI-TOF MS. It also confers the advantage of selectivity as long as the analytes of interest are easily ionized. However, this selectivity places certain limitations on the MALDI-TOF MS technique as a general screening tool, since not all compounds in the extract mixture will necessarily be detected.

There is the potential for MALDI-TOF MS to be used in screening the composition of other types of plant foods, if one is aware of the variation in ionization and fragmentation. In this study, the simple extraction procedure coupled with the speed and reliability of the MALDI-TOF MS analysis provide a powerful combination for comparing and characterizing the flavonol glycoside composition in seedcoats of different almond varieties.

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# Chapter 7

## General Discussion, Conclusions, and Future Research Possibilites

MALDI-TOF MS is fast becoming one of the most significant analytical developments in recent years. The ability to rapidly analyze large thermolabile biomolecules is revolutionizing the fields of biochemistry, medicine, genetics, and many others. A testament to the significance of this technique was the partial award of the Nobel Prize in Chemistry 2002 to one of the pioneers of MALDI-TOF MS technology, Koichi Tanaka (Shimadzu Corp., Kyoto, Japan). Basic research is essential to improving the understanding of where MALDI-TOF MS may be valuable in food science, by providing new approaches to acquire rapid, sensitive, and definitive answers to a number of important questions. This thesis has explored the applicability of MALDI-TOF MS for studying ginsenosides, hydrogenated starch hydrolysates, polysorbate emulsifiers, and almond seedcoat flavonol glycosides.

**Investigation of MALDI-TOF MS for Ginsenoside Analysis.** It is widely recognized, particularly in the area of protein analysis, that MALDI-TOF MS is relatively tolerant of salts, buffers, detergents, and solvents. Less well understood are the factors contributing to the success in MALDI-TOF MS analyses of complex mixtures obtained from foods, which contain many other types of impurities such as sugars, oligosaccharides, fibre, lipids, proteins and peptides, minerals, and other metabolites. In the investigation of MALDI-TOF MS for analyzing ginsenosides, pure standards produced high-intensity peaks. In contrast, ginsenosides partially purified from an extract of American ginseng root powder were not reliably identifiable by MALDI-TOF MS, although their presence in the extract was confirmed by TLC and HPLC analyses. It is not well understood why MALDI-TOF MS works for some compounds in a mixture and not others. Occasionally, some families of compounds are preferentially ionized and suppress the ionization of other families, although in this case no other significant peaks were observed. The intermittent ginsenoside signal in the mass spectrometer suggests that further purification may be required prior to analysis. Future avenues for investigation include a more extensive examination of novel solid-phase extraction cartridges such as the Oasis™ product line from Waters (Milford, MA), or exploring selective solvent extraction techniques to improve the purity of the ginsenoside fraction.

**Characterization of Hydrogenated Starch Hydrolysates by MALDI-TOF MS.** Ideally, to easily characterize and differentiate polymeric mixtures by MALDI-TOF MS, the relative responses should remain relatively constant from spectrum-to-spectrum and should correlate with the percent composition. In the study of hydrogenated starch hydrolysate (HSH) syrups, there was a significant disparity between the syrup composition reported by the manufacturers and the composition determined by mass spectral percent response. Although MALDI-TOF MS has been used successfully to quantify maltooligosaccharides (Wang et al. 1999; Kazmaier et al., 1998), a polymeric carbohydrate series homologous to HSH, the lack of agreement with manufacturer data casts doubt on the validity of this methodology to reliably identify these samples.

Sensitivity in MALDI-TOF MS often depends on the molecular weight of the analytes, thus when analyzing a polymer with a wide mass range, the peak profile may not directly relate to the molecular weight distribution. Furthermore, although MALDI-TOF MS provides more detailed compositional information than any other currently available system, the variability observed between replicate spectra was too great to readily distinguish among HSH samples which differ only slightly in composition. An alternate method of analysis such as high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) may be useful to validate the mass spectrometric results, at least in the low mass range. This technique is more sensitive than HPLC and allows the analysis of carbohydrates without derivatization. However, higher molecular weight oligomers (>DP-10) may be difficult to determine. Further work is also necessary to improve the consistency in spectrum-to-spectrum response.

**MALDI-TOF MS Characterization of Polysorbate Emulsifiers.** MALDI-TOF MS has been used extensively to characterize many synthetic and natural polymers (Nielen, 1999). Analysis of food-grade polysorbate emulsifiers using MALDI-TOF MS provides a level of structural detail unattainable by any other currently available technique. In these complex mixtures, several families of molecules were identified: free ethylene oxide polymers, free and esterified sorbitan- and sorbide-based molecules, and possibly disorbitan derivatives. The spectral complexity, lack of appropriate internal standards, and potential for oligomer discrimination or mass discrimination in polydisperse polymers are potential barriers to quantification of these important compounds. However, the ability of MALDI-TOF MS to easily and rapidly provide a molecular "fingerprint" of polysorbate composition opens possibilities to explore other chemically similar polyoxyethylene polymers such as those of the Brij$^{TM}$ (polyoxyethylene ethers), Myrj$^{TM}$ (polyoxyetheyelene esters), or Triton$^{TM}$ series. Specifically, members of the Brij$^{TM}$ series are easily analyzed by MALDI-TOF MS and produce much simpler mass spectra than polysorbates. A representative example is shown in Figure 7-1. Future investigations to correlate physical properties of these emulsifiers with their chemical compositions could potentially improve the ability to precisely define and predict functional properties of polyoxyethylene emulsifiers.

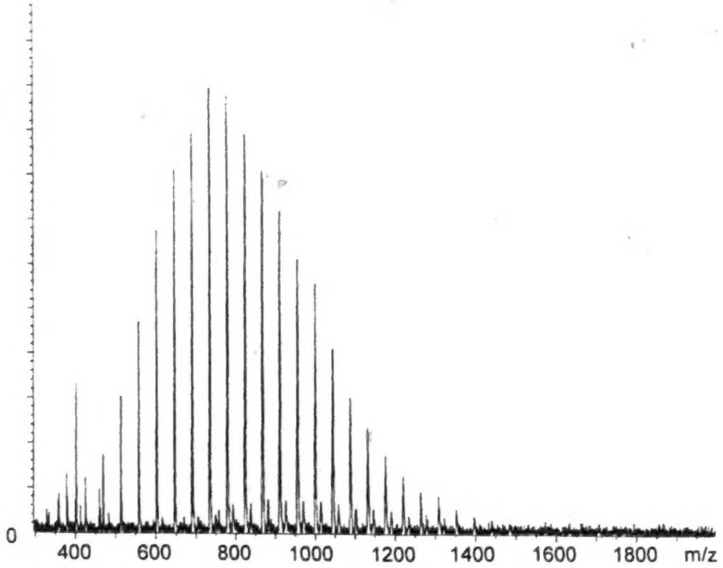

**Figure 7-1**. MALDI-TOF MS spectrum of Brij$^{TM}$ 76 (polyoxyethylene (10) stearyl ether) in 0.01M NaCl with THAP matrix.

**Quantitative MALDI-TOF MS and Screening of Flavonol Glycosides.** A quantitative MALDI-TOF MS protocol was developed for the simultaneous analysis of four flavonol glycosides in almond seedcoats: isorhamnetin rutinoside, isorhamnetin glucoside, kaemferol rutinoside, and kaemferol glucoside. The internal standard rutin (quercetin-3-rutinoside) showed excellent linearity with kaempferol-3-rutinoside and isorhamnetin-3-rutinoside standards. In the simplest quantitative scenario, one signal per analyte would be generated with good resolution. MALDI, although softer than many other ionization methods, still produces multiple ion forms and a degree of fragmentation, often when carbohydrates are involved. Fragmentation of the analytes can further complicate quantitative determination. In this study, because the internal standard and the analytes exhibited similar fragmentation, the fragmentation patterns of rutin were used to approximate the fragmentation patterns of the analytes. However, in cases where an appropriate internal standard which mimics the fragmentation of the analytes is not available, quantification of certain analytes may not be possible. Investigating alternative matrices and sample preparation techniques may present a new approach to obtaining more reproducible spectra. The agreement of the MALDI-TOF MS results with HPLC data suggests that MALDI-TOF MS has potential to be used as a rapid alternative to the widely used but slower chromatography techniques. A more comprehensive study would be necessary to determine with greater confidence the accuracy of the MALDI-TOF MS methodology compared to conventional techniques.

Following development of the quantitative protocol, the method was applied to screen the flavonol glycoside composition of sixteen varieties of almond seedcoats. Quantitative determinations using MALDI-TOF MS have been widely documented and reviewed (Muddiman et al., 1995). However, despite nearly a hundred reports, quantitative MALDI-TOF MS is still viewed with a degree of skepticism. Shot-to-shot and spectrum-to-spectrum variability continue to be the largest barriers to reliable and accurate quantification. In this study, however, triplicate MALDI-TOF MS analyses of each sample were remarkably consistent; the overall variability was 9%, largely attributable to the selection of an appropriate internal standard.

Samples were manually applied to the target and peak intensity data were transferred from the mass spectrometer software (Bruker Daltonics XMASS™ version 5.0) to Microsoft Excel™ for further processing. Recent developments in MALDI-TOF MS technology such as automatic sample deposition by robotic systems, high-throughput analysis of 96 samples at a time, and improvements in data handling software could prove immeasurably valuable to applying MALDI-TOF MS in routine food analysis in general laboratory settings. For example, using a hydrophobic/hydrophilic contrasting MALDI-TOF MS target and a robotic workstation, MALDI-TOF MS has recently been demonstrated to be a powerful tool for high-throughput screening of hemoglobin abnormalities in neonates at a rate of ~100 samples per hour (Kierman et al., 2002). This type of screening capability could easily be applied in many food science areas for rapidly assessing adulteration, validating phytochemical compositions of functional foods and nutraceuticals, ensuring organoleptic quality standards, or monitoring toxin levels, to name a few examples.

**Future Possibilities.** Although the use of MALDI-TOF MS in the food industry is not yet widespread, there is vast potential for many food applications, especially in light of recent technological advances allowing miniaturization (Ekstrom et al., 2000), high throughput analysis (Kierman et al., 2000) and on-line coupling to liquid chromatography (Lab Connections, Northborough, MA). Research is ongoing to improve crystallization homogeneity by using alternative matrices such as ionic liquids (Armstrong et al., 2001), two-phase systems (Kinumi et al., 2000), and solvent-free sample preparation (Trimpin et al., 2001). Mass Technologies Inc. has introduced an atmospheric pressure MALDI (AP/MALDI) source allowing ion production under ambient pressure conditions. Thus, it is readily interchangeable with other ion sources such as electrospray and tends to provide even softer ionization conditions than conventional MALDI (Mass Technologies Inc., Burtonsville, MD). Biorective MALDI probes are newly commercially available from Intrinsic Bioprobes Inc. (Tempe, AZ ) to perform rapid, sensitive, and accurate protein analysis and mass spectrometric immunoassays for selective isolation of proteins from complex biological mixtures. With the explosion of interest in MALDI-TOF MS and the corresponding technological refinements, many of the current limitations to widespread commercialization are being addressed. It may not be long before MALDI-TOF MS is included as a routine instrument in general food analysis laboratories.

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# Appendix I

## Fragmentation Correction Factors for Quantitative Flavonol Glycoside Analysis (Chapter 5)

The fragmentation pattern of an internal standard (rutin) is used to predict the fragmentation patterns of flavonol rutinoside analytes. Because the fragmentation patterns can vary substantially from run to run, a different correction factor must be calculated for each run. Peak heights are considered to be the sum of all of the adduct ions for a particular compound.

1. Calculate the fragmentation ratios (FR) of the internal standard (IS):

$$FR_{1(IS)} = \frac{\text{peak height of aglycone}_{(IS)}}{\text{peak height of rutinoside}_{(IS)}} \qquad FR_{2(IS)} = \frac{\text{peak height of glucoside}_{(IS)}}{\text{peak height of rutinoside}_{(IS)}}$$

2. Assume that the relative responses of rutin fragment ions are the same as those of the analyte (A = isorhamnetin rutinoside or kaempferol rutinoside) fragment ions. Therefore:

   a) Proportion of aglycone response from rutinoside fragmentation (V)

$$V = FR_{1\,(IS)} = FR_{1\,(A)}$$

   b) Proportion of glucoside response from rutinoside fragmentation (W)

$$W = FR_{2\,(IS)} = FR_{2\,(A)}$$

3. Therefore, rutinoside correction factor, **RCF = 1 + V + W**

4. Subtract proportion of response attributable to rutinoside fragmentation to find proportion of response attributable to glucoside.

   a) Proportion of aglycone response from glucoside fragmentation (X)

$$X = \frac{\text{peak height of aglycone}_{(A)} - FR_{1\,(A)}}{\text{peak height of rutinoside}_{(A)}}$$

   b) Proportion of glucoside response in sample (Y)

$$Y = \frac{\text{peak height glucoside}_{(A)} - FR_{2\,(A)}}{\text{peak height rutinoside}_{(A)}}$$

c) Relative glucoside response compared to rutinoside response in mass spectrum (Z)

$$Z = \frac{\text{peak height glucoside}_{(A)}}{\text{peak height rutinoside}_{(A)}}$$

5. Therefore, glucoside correction factor, **GCF = (X+Y)/Z**